ANTI-TUMOR AND PRO-TUMOR INTERACTIONS OF HUMAN NK CELLS IN CANCER

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While natural killer (NK) cells have been classically understood as innate cytotoxic effector cells, a new paradigm has emerged involving NK cells as key immunomodulators in the development of both innate and adaptive immune responses. In particular, NK cells can critically shape the character of anti-cancer immunity through their engagement with dendritic cells (DCs). Thus, understanding the interactions of NK cells with DCs, as well as other cell types in the human tumor environment, is essential to understanding endogenous anti-tumor immune responses and developing effective cancer immunotherapies.

In this work, we show that human NK cells can perform distinct 'effector' and 'helper' activities, which can be uniquely driven by distinct cytokine activation. While IL-2-activated 'effector' NK cells efficiently kill tumor cells and both immature and mature DCs, IL-18-activated 'helper' NK cells instead uniquely potentiate anti-tumor immune responses through DC activation and the enhancement of DC-induced type-1 immunity. These IL-18-activated helper NK cells further recruit DCs, facilitating productive NK-DC interaction, and subsequently collaborate with DCs in promoting chemokine environments conducive to naïve T cell priming in the lymph nodes as well as effector T cell infiltration into peripheral tumor sites.

However, our studies also indicate that, in addition to their desirable anti-tumor type-1-polarizing interactions with DCs, such IL-18-activated NK cells may also have undesirable pro-tumor effects through their IFNγ- and TNFα-dependent hyper-activation of myeloid-derived

suppressor cells (MDSCs), a critical cell population present in most cancers that play a major role in tumor-associated immune suppression and the potential modulation of Th17 immunity. Using MDSCs isolated directly from the malignant ascites of patients with late-stage ovarian cancer, we implicate autocrine COX2/PGE_2 feedback as essential in NK cell-mediated MDSC hyper-activation, highlighting the possibility for therapeutic COX2/PGE_2 axis inhibition in reversing the pro-tumor NK cell-mediated up-regulation of MDSC activity, while preserving or enhancing the anti-tumor NK cell-mediated activation of DCs.

Overall, these studies help to better understand the interactions between NK cells, DCs, and MDSCs in cancer immunity, and identify new targets for the therapeutic manipulation of anti- and pro-tumor NK cell activities for the improvement of cancer therapy.

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PREFACE

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1.0 INTRODUCTION

Despite declines in cancer mortality over the past two decades, cancer remains the leading cause of death among both men and women aged 40-79 and the second leading cause of death in the United States overall, with more than 1.6 million new cases and more than 580,000 deaths projected to occur in the US in 2013 [1]. Conventional combination treatment with surgery, radiotherapy, and chemotherapy has shown success in reducing tumor burden, but has been less effective in eliminating residual cancer and preventing disease recurrence, limiting the overall effectiveness of these approaches as definitive cancer therapy. Therapeutic stimulation of the immune system to better recognize and kill cancer cells has been proposed to overcome these limitations [2], seeking to harness the sensitivity, specificity, and durability of the protective immune response evolutionarily honed, in part, to detect and eliminate aberrations associated with cancer. Nevertheless, despite the simplicity of this overall rationale, incomplete understanding of the complex nature of the anti-tumor immune response continues to remain a significant obstacle to immunotherapeutic development, including limited knowledge of the optimal activation and interaction of key immune players necessary for the induction, amplification, and execution of robust anti-tumor immunity, as well as the critical feedback mechanisms inhibiting these responses. It has also become increasingly appreciated that the tumor environment itself represents a unique immunologic atmosphere incorporating both

physiologic and pathologic mechanisms for its survival and expansion, the understanding of which, particularly in the human setting, is essential to developing effective therapy.

This thesis aims to understand key relationships in the initiation and development of anti-tumor immune responses in the context of human cancer. In it, natural killer (NK) cells and their interaction with dendritic cells (DCs) are identified as key initiators and propagators of adaptive anti-tumor immunity, and the unique cytokine- and chemokine-driven regulation of these activities are described. This work further explores these concepts in the human tumor environment, including identification of the critical role of myeloid-derived suppressor cells (MDSCs) in determining tumor environment-associated immune outcomes, and the contrasting stimulatory and suppressive interface of NK cells with DCs and MDSCs. These studies provide comprehensive new insights into NK cell-mediated regulation of anti-tumor immune responses as well as broader implications for the enhancement of type-1 immunity in cancer therapy.

1.1 IMMUNITY AND HUMAN CANCER

While the immune control of cancer has long been postulated [3, 4], it has only relatively recently been established as a key mechanism for anti-tumor surveillance. This paradigm has been driven by seminal work describing the critical role of IFNγ and lymphocytes in protection from tumors in mouse models [5-8]. Importantly, these animal studies have been supported in the human setting by the correlation between clinical outcome and the quantity and character of the intratumoral immune cell infiltrate [9], as well as by the clear clinical effect of established and emerging cancer therapeutic agents targeting the modulation of the immune system [10-20]. Nevertheless, while these findings provide evidence of distinct clinical benefit and strong proof-

of-principle for the involvement of the immune system in cancer control, these findings also emphasize the emerging awareness that a complete understanding of the anti-tumor response necessarily depends on appreciating detailed characteristics of immune processes within the actual human tumor environment itself.

1.1.1 Immune environment of human tumors

In situ analysis of human tumors has revealed considerable heterogeneity in the numbers, types, functional orientations, and distributions of immune cells between patients and between tumor types. Correlation of these intratumoral immune parameters with clinical outcomes has allowed an enhanced understanding of key cells and processes that may critically determine tumor control versus tumor progression. An analysis by Fridman and colleagues of 124 published studies correlating immune cell infiltration of diverse human tumors with clinical results [21] revealed that high numbers of infiltrating $CD3^+$ T cells, $CD8^+$ cytotoxic T cells (CTLs), and $CD45RO^+$ memory T cells are clearly associated with longer disease-free and overall survival. Analysis of $CD4^+$ T cell subsets further revealed a strong association between Th1 cell infiltration and good clinical outcome. Analysis of the prognostic significance of other $CD4^+$ T cell subsets was less clear, due potentially to imprecise lineage-defining markers (such as the expression of CD25 and FOXP3 on both regulatory and activated effector T cells) or variation between different tumor types. Nevertheless, a multitude of studies have identified regulatory T (T_{reg}) cell infiltration as a poor prognostic marker for melanoma, breast, ovarian, lung, pancreatic, hepatocellular, and renal cell cancers, with the significance of Th2 and Th17 cell infiltration varying by tumor type. Overall, however, these studies clearly support the induction

of robust type-1 immunity and the promotion of Th1 and CTL activities directly within the tumor environment as a key goal of cancer immunotherapeutic approaches.

These *in situ* analyses of human tumors have also clearly revealed that the tumor immune environment is a complex array of immune cells beyond T cells, including important populations of NK cells [22, 23] and DCs [24, 25], as well as significant suppressive myeloid populations like MDSCs [26, 27] (significance of these cell populations are further discussed below). Although interaction between these cell types (strongly suggested by their proximate localization within the tumor environment [21]) are likely to play a large role in defining the intratumoral immune context, including significant effects on T cell localization and activity, these interactions still remain unclear.

1.1.2 Chemokines

The chemokine system has emerged as an essential regulator of the spatial and temporal events governing both the priming and effector phases of anti-tumor immunity, as well as immune processes within the tumor environment itself. This includes the localization of DCs in peripheral tumor tissues for antigen acquisition, subsequent migration of antigen-carrying DCs to regional lymph nodes (LNs), concurrent trafficking of naïve T cells to the LN and their intranodal association with DCs for efficient priming, and the migration of activated effector cells back to peripheral tumor sites.

Resident tissue macrophages and dendritic cells play a key role in initiating immune responses through their recognition of damage- and pathogen-associated molecular patterns (DAMPs and PAMPs) using Toll-like receptor (TLR) and other pattern-recognition signaling, and their subsequent secretion of chemokines including CCL3-5 and CXCL8 [28]. These

chemokines are critical for the recruitment of neutrophils as well as additional macrophages and immature DCs [29], expanding the potential for antigen acquisition. Interestingly, NK cells similarly exhibit a robust capacity to integrate multiple danger-, damage-, and pathogen-associated signals, including through TLRs as well as the direct recognition of tumor and viral-infected targets using a large panel of activating and inhibitory receptors (see ‘NK cell activation’ below). Whether NK cells may have a role in the chemokine-driven initiation of immune responses remains unclear.

The maturation of antigen-carry DCs is accompanied by a switch in chemokine receptor expression from CCR1, CCR2, CCR5, and CXCR1 to the high expression of CCR7 [30]. The expression of CCR7 confers DC responsiveness to the chemokines CCL19 and CCL21, expressed within primary and secondary lymphoid organs by stromal cells and other mature DCs, facilitating DC trafficking into the LN [31]. The CCR7-CCL19/CCL21 axis is also critical for the entry of naïve T cells into the LN [32], as well as for the subsequent close intranodal localization of DCs and T cells [33]. Dynamic cellular activities conducive to efficient adaptive immune priming, such as T cell motility and scanning behavior, are also promoted by the CCR7-CCL19/CCL21 axis [34-37]. DC priming subsequently induces CCR7 downregulation on T cells, facilitating their egress from the LN, as well as differential T cell acquisition of peripheral-homing chemokine receptors driven by the polarizing environment, directing activated T cells to peripheral sites of inflammation. For instance, under Th2-polarizing conditions, T cells upregulate CCR3, CCR4, and CCR8, while under Th1-polarizing conditions, T cells upregulate CXCR3 and CCR5 [38].

As described above, the quantity and quality of immune cell infiltration into the tumor environment have been increasingly recognized as vital components of both spontaneous and

therapy-induced anti-tumor immune control [9, 39-41]. Chemokines have been identified as key modulators of this process, with the potential to exquisitely define the character of the immune cell infiltrate in the tumor environment [42]. Importantly, the chemokines CXCL9, CXCL10, CCL5, and CX3CL1 have been implicated in the attraction of type-1 memory and effector T cell (T_{eff}) subsets, which are central to efficient anti-tumor responses and are associated with prolonged disease-free and overall survival [43-46]. In contrast, other chemokines present in the tumor environment, including CCL22, have been implicated in the attraction of T_{reg} cells, associated with a poor clinical outcome in many cancers [47-49]. Thus, the tumor environment represents a complex chemokine network with dramatic consequences for the outcomes of intratumoral immunity, providing key targets for therapeutic 'conditioning' of the tumor environment for anti-tumor effector cell entry. Activated DCs have been shown to produce a number of chemokines [28, 30] and can play an important role in defining the chemokine environment in human cancer [50], but the regulation of this production, particularly the role of specific cellular interactions, are not yet fully known.

1.2 NATURAL KILLER CELLS

NK cells were first characterized histologically and functionally as large granular lymphocytes with the unique ability to kill target cells in the absence of prior sensitization [51, 52]. Early refinements to this characterization came in studies investigating the rejection of bone marrow allografts in mice [53, 54], leading to the formulation of the seminal 'missing self' hypothesis [55] proposing that NK cells would kill any target lacking self-major histocompatibility complex (MHC) class I molecules. This provided an initial framework to understand the regulation of NK

cell effector activity. Since those early observations, it has become clear that NK activation is a much more complex, and still incompletely understood, phenomenon, involving the integration of signals from a multitude of activating and inhibitory receptors engaging MHC class I molecules, MHC class I-like molecules, and other molecules unrelated to MHC, as well as from an extensive array of cytokines distinct from, but related to, target recognition (see 'NK cell activation' below). It has also become appreciated that NK cells can play important roles in immune responses separate from their cytotoxic capacity, acting as important immunomodulatory cells engaged in reciprocal interactions with many cell types, including dendritic cells, macrophages, T cells, and endothelial cells (see 'NK cell functions' below). Here, we introduce the evolving modern concepts of NK cell activation and NK cell function, with particular emphasis on their role in anti-tumor immunity.

Human NK cells are typically defined phenotypically within the lymphocyte population by the absence of CD3 (thereby excluding T cells) and the expression of CD56, found on NK cells and a minority of T cells [56], and also more recently by the expression of NKp46, a highly-conserved natural cytotoxicity receptor [57-59]. It is important to note however that NK cells actually represent a heterogeneous population of cells with distinct phenotypic and functional subsets. Approximately 90% of NK cells in human peripheral blood have low expression of CD56 and are typically associated with enhanced perforin expression and cytotoxic activity [60]. In contrast, approximately 10% of circulating NK cells (but markedly enriched in secondary lymphoid tissue in the steady state [61, 62]) express high surface levels of CD56, and have been associated with high production of cytokines and chemokines and lower cytotoxic capacity [63]. However, despite this classical distinction of NK cell subset activities, recent findings have indicated that the $CD56^{dim}$ subset can also be a major producer of cytokines and

chemokines, particularly at early time-points after activation [64, 65]. The specific regulation of these distinct cytotoxic versus cytokine/chemokine-secreting capacities of $CD56^{dim}$ NK cells remains to be determined. Furthermore, although a number of studies have suggested that the transition from $CD56^{bright}$ to $CD56^{dim}$ NK cell populations marks progression in a continuum of NK cell differentiation [66, 67], these populations likely play distinct roles in immune responses, and the distinct activities and activation responses of these different subsets warrants continued investigation.

1.2.1 NK cell activation

Despite their initial characterization as spontaneous effector cells, resting human peripheral blood NK cells in fact exhibit poor effector function [68]. Likewise, in mice, resting splenic NK cells are poorly cytotoxic due to reduced levels of granzyme B and perforin, which are only induced after cytokine stimulation or viral infection [69]. Thus, it has become increasingly apparent that activation is required under most circumstances for the full acquisition of NK cell effector functions [70]. An extensive body of work now indicates that this activation is achieved only through the proper integration of multiple signals, including an extensive array of both cell surface and soluble factors.

Unlike T and B cells possessing a single antigen receptor dominating their activation, NK cell detection and discrimination of target cells from healthy ‘self’ cells, as well as the recognition and interaction with other immune cells, is significantly determined by the dynamic equilibrium of signals provided by a vast combinatorial array of activating and inhibitory receptors. Indeed, ligation of any single receptor alone, with the exception of CD16, is not able to induce cytolytic activity or cytokine secretion, which instead relies on combinations of

receptor cross-linking [68]. Activating NK cell receptors typically recognize ligands presented by cells in distress. These receptors importantly include NKG2D, recognizing the cellular stress-induced non-classical MHC class I molecules MICA/B and the MHC class I-related ULBP molecules rarely expressed on healthy cells, but induced by viral infection, DNA damage, and transformation [71, 72]. The NKG2D receptor has been heavily implicated in NK cell control of tumors, including in spontaneous mouse models of lymphoma and prostate carcinoma [73]. The natural cytotoxicity receptors (NCRs), including NKp46, NKp30, and NKp44, are also a group of key NK cell activating receptors, recognizing such ligands as BAT3 and B7-H6 on tumor cells [74, 75] and viral hemagglutinins [76, 77]. DNAM-1 and 2B4 likewise represent important NK cell activating receptors, recognizing the CD112/CD155 and CD48 ligands, respectively [78-80], and have shown important roles in tumor cell elimination [81-83]. CD16, mediating antibody-dependent cellular cytotoxicity of opsonized targets, can also play a significant role in NK cell activation [68, 84], and may be a dominant component of the anti-tumor activity mediated by therapeutic antibody treatment [85]. Importantly, the anti-tumor activity conferred by these receptors is most often the result of receptor cooperation, such as the involvement of NKG2D, NKp46, and DNAM-1 in killing of multiple myeloma [86] and the NCR- and DNAM-1-mediated killing of melanoma [87], providing both synergistic effects among receptors [65, 68] as well as the capacity to recognize diverse tumors with often variable expression of individual receptor ligands [86, 87]. NK cells are also capable of recognizing infectious non-self ligands through the expression of several TLRs [88], although the activation of NK cells by TLR ligands may be more efficient via accessory cell involvement *in vivo* [89, 90]. These findings indicate that signals from multiple NK cell activating receptors cooperate in the detection of cellular stress from damage, infection, or transformation.

To prevent inappropriate activation of NK cell functions, the signals from NK cell activation receptors are balanced by signals from a wide array of inhibitory receptors, many of which recognize MHC class I molecules constitutively expressed on most healthy cells in the steady-state, but which may be reduced or lost under stress. These receptors in humans include the killer cell immunoglobulin-like receptors (KIRs) and the lectin-like CD94-NKG2A heterodimers containing immunoreceptor tyrosine-based inhibition motifs (ITIMs), which mediate inhibitory signals that in some cases can override activation stimuli [91, 92]. NK cells may also detect other constitutive self-signals apart from MHC class I through a variety of other inhibitory receptors, including NKR-P1A and LAIR-1 [93, 94]. These studies indicate close regulation of NK cell activation to ensure tolerance of healthy self. Indeed, the engagement of MHC class I-specific inhibitory receptors has been shown to play a critical education process to ensure self versus altered-self discrimination, in which inhibitory receptor recognition of self MHC class I molecules are needed to 'license' or 'arm' NK cells for strong immune reactivity [60, 95, 96]. These findings highlight the complex interplay that exists in NK cell ligation of stimulatory and inhibitory receptors, which carefully regulates full effector activation.

In addition to the ligation of NK cell surface receptors, the activation of NK cell responses also depends significantly on the cytokine microenvironment. IL-2 is a classical promoter of NK cell proliferation, cytotoxicity, and to some extent, cytokine secretion [97], provided potentially *in vivo* by $CD4^+$ T cells during co-localization in the lymph nodes [61]. Several other cytokines, including type I interferons, IL-18, IL-12, and IL-15 have also been described as potent activators of NK cell effector function [98], potentially provided during interactions with other cell types including dendritic cells and macrophages [99]. Cytokine activation has also been shown to closely cooperate with cell surface receptor ligation in NK cell

activation, such as in the coupled signaling between IL-15 and NKG2D [100]. How these different cytokine factors may differentially regulate specific and distinct NK cell functions, however, continues to remain unclear.

Type I interferons and IL-18 are particularly intriguing from the standpoint of the early activation of NK cells. Type I interferons are well known to be elaborated early in the course of viral infection [101], and have also recently been shown to play a key role in initiating spontaneous anti-tumor immune responses *in vivo* [102, 103]. IL-18 is constitutively expressed by multiple mucosal and barrier cell types, especially by keratinocytes in the skin and epithelial cells in the gut and lung [104-106]. Secretion of active IL-18 is also known to critically depend on inflammasome-regulated capase-1 activation downstream of infection or recognition of a wide variety of danger signals associated with cell damage and cell death, such as extracellular ATP and free uric acid [107]. Thus, both type I interferon and IL-18 represent early indicators of the need for an immune response, and how these factors may drive NK cell roles specifically in the initiation of immune responses is an intriguing question that remains to be explored.

1.2.2 NK cell functions

While identified early on by potent lytic ability against tumor and viral-infected targets, NK cell functions are now appreciated to encompass a multifaceted array of cytotoxic and non-cytotoxic activities driven, as described above, by diverse activation stimuli. Although these lytic and non-lytic functions, reviewed below, can occur in concert, evidence also indicates that these activities are in fact distinct and may occur independently under many circumstances [108-110], the selective regulation of which remains to be fully elucidated.

1.2.2.1 NK cell 'effector' activity

As their name suggests, NK cells can potently kill target cells through a diversity of mechanisms, representing a key function of NK cells in cancer immunity. Primarily, NK cells employ a complex and highly-regulated lytic response involving the polarization and localized secretion of lytic granules containing perforin and granzymes at the NK/target interface [111], progressing through a sequential step-wise process of synapse formation between effector and target, rapid cytoskeletal rearrangement with reorientation of the microtubule-organizing center and cytotoxic granules toward the synapse, and granule docking and fusion at specialized secretory domains [112-114]. NK cell expression of death receptor ligands FasL and TRAIL has also been implicated as an important mechanism for killing tumor targets expressing the cognate receptors [115-118], and NK cells have also been shown to kill via the release of exosomes containing perforin and death receptor ligands [119] or through long-distance lytic synapse formation utilizing membrane nanotubes [120]. It is important to note that NK cell lytic interactions are not limited to tumor or infected targets, and have been shown to be capable of killing syngeneic activated T cells [121], other NK cells [122], and multiple myeloid cell types, importantly including DCs (see 'NK-DC cross-talk'), with great potential for influencing downstream immune responses.

1.2.2.2 NK cell 'helper' activity

In addition to their cytotoxicity, it has become increasingly appreciated that NK cells perform equally-important non-cytotoxic immunomodulatory functions that can often be quite distinct from their killing capacity, with significant consequences for anti-tumor responses. These functions are significantly mediated by IFNγ, a cytokine with pleiotropic effects on anti-tumor immunity, with NK cells serving as a critical early source of this cytokine in the developing

immune response [123, 124]. These effects include direct induction of tumor cell apoptosis, direct suppression of tumor cell proliferation and metastasis, inhibition of tumor angiogenesis, and upregulation of MHC class I and antigen processing machinery [125], and NK cell-derived IFNγ has also been recently shown to induce M1-polarized macrophages contributing significantly to innate tumor immunoediting [125]. Although IFNγ secretion is the cytokine most often associated with NK cells, NK cells have also been demonstrated to secrete a number of other factors, including TNFα, GM-CSF, IL-13, IL-10, and multiple chemokines [63, 65, 126, 127], although the specific regulation of the production of these factors remains unclear. Perhaps one of the most important immunomodulatory functions of NK cells is the shaping of adaptive immunity, mediated in close collaboration with DCs, involving the maturation and activation of DCs and the polarization of T cell responses (discussed in detail below in 'NK-DC cross-talk'), with IFNγ, TNFα, and cell-surface molecules, such as NKp30, playing key roles [128]. However, despite the centrality of this process to bridging innate danger recognition with adaptive effector responses, critical details of these interactions remain to be elucidated.

1.2.3 NK cells and cancer

Although the exceeding rarity of human NK cell-selective deficiencies has limited the precise characterization of the human *in vivo* requirement for NK cells in anti-tumor immunosurveillance, a seminal epidemiologic study by Imai and colleagues following a cohort of 3625 participants over a period of 11 years demonstrated an association between high NK cell activity in peripheral blood and reduced cancer risk [129], suggesting an important link between NK cell activity and tumor control. Furthermore, NK cell infiltration into human tumors has been correlated with improved prognosis for multiple human cancers, including lung, colorectal,

gastric, liver, and renal [23, 130-133], although interpretation of these studies is complicated by their reliance on the CD57 surface marker also expressed by activated CTLs. More recent studies using the NKp46 marker, more specific for NK cells, have shown mixed results, with increased intratumoral NK cells associated with an improved prognosis in early stage breast cancer [134] but not in non-small-cell lung cancer (NSCLC) [22]. These equivocal results may possibly be explained by recent findings indicating potential functional defects present in intratumoral NK cells. These include downregulation of multiple NK cell activating receptors (such as CD16, NKp46, NKp30, NKp80, DNAM1, 2B4, and ILT2) [22, 135-137] as well as upregulation of NK cell inhibitory receptors like NKG2A [138, 139], preventing NK cell activation and the cytolysis and secretion of IFNγ needed for effective anti-tumor activity. Ligands for NK cell activating receptors, such as NKG2D, may also be shed from the surface of tumors, leading to decreased NK cell recognition of tumors as well as reducing NK cell receptor expression [140, 141]. Furthermore, indoleamine 2,3-dioxygenase (IDO), prostaglandin E_2 (PGE_2), TGF-β_1, and other factors expressed within the tumor environment have also been shown to contribute to intratumoral NK cell impairment [142, 143]. These results indicate that NK cell are likely to play a protective role in anti-tumor immunity, but may be compromised in advanced cancer. This highlights the critical need to address paradigms of therapeutic NK cell activation both in the specific context of tumor-associated NK cells from cancer patients as well as in the context of suppressive mechanisms likely to exist in the human tumor environment. In particular, although the interaction of NK cells with critical suppressive populations, such as MDSCs, have been suggested by a limited number of mouse studies [144] and the close localization of NK cells and MDSCs within human tumors [21], current knowledge, particularly in the human context, of this interaction is lacking and represents a critical area of needed research.

1.3 DENDRITIC CELLS

DCs are ubiquitous tissue-resident cells specialized to capture and process antigens, both associated with self as well as with pathogen infection or tumor transformation [145]. In the steady state, DCs in peripheral tissues are immature, capable of efficiently capturing antigens but expressing low levels of co-stimulatory molecules and a limited capacity to secrete cytokines [145]. Immature DCs presenting self-antigens can induce immune tolerance via deletion or anergy of self-reactive T cells, or through the expansion of regulatory T cells [146, 147]. However, upon activation and maturation through a variety of mechanisms [148], including the sensing of pathogen-related products, factors associated with tissue-damage, or instructive signals provided by other immune cells detecting transformation or infection, they act as carriers of antigenic and contextual inflammatory information from the periphery to draining lymph nodes (LNs). Here, they act to prime naïve T cells to antigen-specific and context-dependent effector function, providing the antigen-specific ‘signal 1’ and co-stimulatory ‘signal 2’ through the increased expression of MHC class II and co-stimulatory molecules [149]; the polarizing ‘signal 3’ through the secretion of specific cytokines regulating the balance between type-1, type-2, and type-17 effector mechanisms [150, 151]; and cell-homing ‘signal 4’ instructing effector cell migration to relevant infected or transformed tissues [152]. The additional ability of activated DCs to stimulate different arms of cellular immunity beyond the T cell compartment, particularly natural killer (NK) cells [153], may further induce diverse responses against potentially distinct, non-overlapping features of cancer alteration. Thus, their ability to directly recognize signals elaborated by infection and tissue distress, as well as integrate signals provided by other innate cells, and translate those signals into the development and enhancement of adaptive and innate responses place DCs as central agents of immunity. Indeed, DCs have been

shown to be essential for the generation of anti-tumor immunity *in vivo* [102, 103], and thus represent key targets for therapeutic antigen delivery and immune induction against cancer.

1.3.1 DCs and cancer

Although the diversity and non-exclusivity of immunohistochemical markers used in the correlation of tumor- and LN-infiltrating DCs with clinical outcome must be interpreted with some caution, tumor-infiltrating DCs (TIDCs labeled with S-100, DC-LAMP, CD83, and/or CD1a, among other markers), particularly mature TIDCs, have been associated with improved prognosis for many cancers [154], suggesting the ability of DCs to mature in the tumor environment and effectively activate adaptive immunity toward tumor-associated antigens. For instance, in melanoma, higher density of mature DC-LAMP$^+$ DCs in both primary tumors and draining lymph nodes was associated with prolonged survival, and was also correlated with reduced tumor thickness and enhanced infiltration of activated T cells into the tumor environment [155, 156]. Similarly, in patients with breast carcinoma, higher numbers of mature CD83$^+$ TIDCs, but not immature CD1a$^+$ TIDCs, had independent prognostic relevance, correlated with longer relapse-free and overall survival as well as reduced lymph node metastasis [157]. These clinical findings have been supported by early proof-of-principle mouse studies demonstrating that adoptive transfer of mature tumor-loaded DCs could induce protective T cell and NK cell responses [153, 158]. And indeed, more recent studies have also shown the essential role of endogenous DCs in promoting spontaneous T cell responses against developing tumors [102, 103], as well as the ability of TIDCs to act as competent and therapeutically-targetable antigen presenting cells for the induction of protective anti-cancer immunity [159].

However, as described above, DCs are critical regulators of both immune activation and immune tolerance, and as tumors most often develop in the absence of danger signals typically associated with acute, pathogen-induced inflammation, DCs presenting antigen from the tumor environment frequently promote tolerance toward these epitopes [160]. This is compounded by active suppressive mechanisms evoked by the tumor environment to subvert DC-mediated immunity through interference with DC differentiation and functional maturation, evidenced by observations of endogenous DC dysfunction in patients with many cancers [154]. For instance, VEGF elaborated in the tumor environment has been shown to inhibit DC differentiation [161, 162], with clinical antibody-mediated blockade of VEGF in lung, breast, and colorectal carcinoma patients shown to reduce the accumulation of immature progenitor cells and increase DC numbers and function in peripheral blood [163]. IL-6 and M-CSF have also been shown to switch the differentiation of monocytes from DCs to macrophages, interfering with efficient DC-mediated anti-tumor T cell priming [164]. The tumor environment has further been shown to inhibit DC maturation, critical in determining the balance between immune stimulation and immune tolerance induced toward DC-carried tumor antigens. For instance, IL-10, commonly found in the tumor environment [165], has been shown to impair expression of maturation markers and co-stimulatory molecules such as CD83 and CD86, and to promote antigen-specific T cell anergy [166]. Indeed, IL-10 blockade was found to enhance the expression of CD83, CD80, CD86, and MHC molecules, production of TNF and IL-12, and T cell activation and priming toward type-1 responses [167].

It is clear, however, that not only maturation but also the specific maturational context is critical in determining the outcome of DC-driven immune responses. For instance, DC maturation in the presence of prostaglandin E_2 (PGE_2), an inflammatory mediator over-produced

in chronic inflammation and cancer in concert with the key regulator of its synthesis, cyclooxygenase-2 (COX2) [168], has been shown to strongly inhibit DC production of IL-12, driving DC-mediated T cell differentiation away from Th1 and toward alternative Th2 and Th17 pathways [169-171]. PGE_2-driven DC maturation has also been shown to inhibit NK cell activity via the reduction of IL-12 and IL-18 [172], as well as to limit the attraction of effector T cells and NK cells and promote the recruitment of regulatory T cells through the diminished production of CXCR3 and CCR5 ligands and the enhanced production of CCL22, respectively [172, 173]. In contrast, DC maturation in the presence of other stimuli (including IFNγ) can induce DC polarization toward high production of IL-12 and the priming of Th1 and CTL responses [174, 175], with the additional involvement of IFNα and TLR ligands like poly-I:C helping to regulate the recruitment of effector T cells [173, 176]. Furthermore, while multiple stimuli including IL-1β/IL-6/TNFα/PGE_2, LPS, poly-I:C, CD40L, and anti-FcγRIIB antibody were shown to similarly induce DC maturation, based on upregulation of CD83, CD80, CD86, and HLA-DR expression, DCs matured in the presence of IL-1β/IL-6/TNFα/PGE_2 demonstrated a significantly enhanced capacity to induce $CD25^+FOXP3^+$ T_{reg} cells and potent immune suppression [177]. Collectively, these data indicate that the context in which DCs mature play a decisive role in determining their immunogenicity in the tumor setting. Thus, it is evident that while DCs can be key promoters of potent anti-tumor immune responses, fully understanding their conditions of activation, as well as prospective avenues for manipulation, will be critical to maximizing their potential for cancer immunotherapy.

1.3.2 NK-DC cross-talk

As described above, NK cells and DCs both play critical early roles in the defense against infection and cancer, recognizing danger signals via complementary and partially-overlapping systems and acting at the forefront of the immune response. Pioneering work by Fernandez and colleagues provided the first evidence of NK-DC interactions *in vivo* as a component of effective anti-tumor immune control [153]. Since that time, a considerable body of work has revealed the close localization of NK cells and DCs within peripheral sites of inflammation and tumor [178-181] as well as within secondary lymphoid organs [182-184], and a complex bi-directional cross-talk between NK cells and DCs has emerged as an important contributor to the development of both innate and adaptive responses to diverse immune challenges [185-188].

Indeed, although DCs are known for their central role in T cell priming and the activation and regulation of adaptive immune responses, DC activation of NK cells has also been shown to be an important component of human *in vitro* [189-191] and mouse *in vivo* anti-tumor responses [153, 192]. While NK cells were first characterized by an ability to kill without prior activation, priming by DCs is now appreciated to be an important mechanism for inducing full NK cell activation, mediated through both soluble cytokine-dependent and cell contact-dependent processes. IL-12 has been repeatedly observed to induce NK cell proliferation, cytotoxicity, and IFNγ secretion [182, 193], and IL-15, originally characterized for its importance in NK cell development, has also been implicated in NK cell effector activation, particularly through DC surface 'trans'-presentation by IL-15Rα [194-196]. Many other factors have also been suggested to have a role in DC activation of NK cells, including IL-18 and type I IFNs [185, 197], membrane-bound TNF [195], Notch ligands [198], and CX_3CL1 [199]. The ability of NK cells under certain patterns of activation to induce DC production of the above-described factors

suggests the possibility for potent feed-forward interactions between NK cells and DCs in the expansion of immune responses, and thus represents an attractive potential target for cancer therapeutic approaches. A complete understanding of the regulation and mechanisms of this relationship, however, remains lacking.

Reciprocally, activated NK cells can play a critical 'helper' role in shaping innate and adaptive immune responses through their modulation of DC function [200]. As mentioned above, type-1 immune responses, dominated by the activation of Th1, CTL, and NK cell responses, are critical for effective surveillance against tumor development and are driven significantly by DC-provided signals [150], with production of IL-12p70 serving as an essential factor [201]. Our group and others have demonstrated that NK cells, following recognition of MHC class I^{low} targets expressing ligands for NK activating receptors and/or exposure to various soluble mediators, including type I interferons, IL-2, and IL-18, can mediate DC activation via TNFα, IFNγ, and cell-to-cell contact-dependent signals, including NKp30 [128, 189, 190, 202, 203]. Such NK cells demonstrate the ability to mature DCs toward high expression of co-stimulatory molecules and promote high IL-12-producing, type-1-polarized DCs with an enhanced capacity to induce anti-tumor Th1 and CTL responses, even in the absence of $CD4^+$ T cell help [204]. NK cells have also been shown to be capable of driving type-1 responses by triggering the differentiation of monocytes into DCs [205, 206]. Collectively, these data indicate that NK cells not only serve as effector cells benefitting from DC activation, but play key roles in activating, broadening, and directing DC-mediated immune responses.

In addition to the promotion and polarization of DC maturation, activated NK cells, particularly those expressing the CD94/NKG2A inhibitory receptor but lacking inhibitory killer Ig-like receptors (KIRs) specific for self-HLA-class I alleles [207], also demonstrate the ability

to kill immature (i)DCs *in vitro* and *in vivo* [189, 208, 209]. A number of different mechanisms have been proposed to mediate NK cell killing of iDCs, including recognition of iDCs via the NKp30, NKp46, and DNAM-1 activating receptors on NK cells [191, 210, 211], and iDC cytolysis via perforin, granzyme, FasL, and TRAIL-dependent mechanisms [208, 212]. Besides iDCs, NK cells have also been implicated in the lysis of other types of myeloid APCs, including resting microglial cells [213] and activated, but not resting, macrophages [214]. Although the direct significance of this NK cell-mediated killing of APCs is still unclear due to the limitations in the experimental models used, this process may act as a potential suppressive mechanism providing negative feedback control over immune activation. Distinct immune synapses have been reported in differentially-mediating lytic and non-lytic NK cell functions during their reciprocal interaction with macrophages [214], and a unique DC-NK 'regulatory' synapse has been described governing the IL-15Rα-mediated survival of NK cells that is qualitatively different from the classical NK synapses formed in association with MHC class I^{low} targets [215]. Overall, these data indicate that NK cell-mediated activation as well as NK cell-mediated killing both represent major outcomes of NK cell interaction with DCs, although the determinants and regulation of these differential outcomes remain unclear.

1.4 MYELOID-DERIVED SUPPRESSOR CELLS

MDSCs are a heterogeneous population of myeloid progenitor cells and activated immature myeloid cells (iMCs) that were first identified in cancer [216, 217], but are now known to also play key regulatory roles in many other pathologic settings, including infection [218-220], autoimmunity [221, 222], and traumatic stress [223]. While iMCs in healthy individuals quickly

differentiate into mature granulocytes, macrophages, or DCs, these pathologic conditions partially block this iMC differentiation and instead contribute to the expansion of MDSCs [224]. The involvement of MDSCs in cancer, as well as their complex phenotypic characterization and functional activities, are reviewed below.

1.4.1 MDSCs and cancer

MDSCs are enriched in the setting of most human cancers and animal tumor models and play a critical role in the establishment and maintenance of an immunosuppressive tumor microenvironment [225, 226]. In healthy individuals, iMCs are non-suppressive, constituting about 0.5% of PBMCs in humans [227], and in mice, they represent about 20-30% of cells in the bone marrow, 2-4% of cells in the spleen, and are absent from the lymph nodes [224, 228, 229]. However, tumor-associated activation results in an up to ten-fold increase in MDSCs in the blood of cancer patients [227, 230], and these cells may comprise up to 50% of splenocytes [224] and lymph node cells in tumor-bearing mice [228]. MDSCs have been isolated from the blood of patients with almost all forms of cancer, including cancers of the colon, lung, breast, liver, prostate, skin, brain, kidney, and many others [231], with the high frequency of these cells often correlating with increased tumor burden, radiographic progression, and poor prognosis [232, 233]. Importantly, MDSCs have also been shown to be profoundly enriched within the human tumor microenvironment [26, 27, 234], both infiltrating and surrounding tumor beds in the core as well as at the invasive margin [21]. These cells thus represent a critical determinant of intratumoral immune responses through their close association with immune cells and their potent suppressive ability, described in detail below (see ‘MDSC functions’).

1.4.2 MDSC phenotype and plasticity

MDSCs show significant differences in phenotype depending on the pathologic condition and tissue specified [228]. The term MDSC comprises cells of myeloid origin at earlier stages of their differentiation (i.e. myeloid progenitor cells and iMCs) that typically do not express or express only limited levels of mature myeloid cell markers [235], and demonstrate either granulocytic or monocytic morphology. MDSCs are identified in mice by co-expression of CD11b and Gr1 markers, as well as by the expression of CD115 (c-fms; receptor for M-CSF) [236, 237], CD16 and CD32 (receptors for Fcγ), IL-4Rα [237], and low levels of CD80 [238]. Murine monocytic MDSCs, at least in some tumor models, express higher levels of CD54, F4/80, IL-4Rα, CD115, and Ly6C [236, 237, 239], and express elevated levels of both arginase 1 (Arg1) and inducible nitric oxide synthase (iNOS), whereas granulocytic MDSCs express high levels of Ly6G and contain significant levels of Arg1 [228, 239, 240]. Initial studies demonstrated at least two distinct cellular fractions within the heterogenous population of MDSCs according to Gr1 brightness: a $Gr1^{high}$ population mainly composed of immature and mature granulocytes, and a $Gr1^{int}$ population comprising monocytes and other immature myeloid cells. More recently, however, MDSCs have been divided into three populations according to Gr1 brightness: polymorphonuclear MDSCs (PMN-MDSCs; $CD11b^{+}Gr1^{hi}Ly6G^{+}Ly6C^{low/int}$), mononuclear MDSCs (MO-MDSCs, $CD11b^{+}Gr1^{int}Ly6G^{-}Ly6C^{hi}$, $F4/80^{+}CD115^{+}7/4^{hi}CCR2^{hi}$), and $Gr1^{low}$ MDSCs [239, 241, 242]. Under this scheme, MO-MDSCs and a subpopulation of $Gr1^{low}$ MDSCs ($Ly6G^{-}Ly6C^{+}F4/80^{+}SSC^{low}$) have come to jointly represent monocytic MDSCs, characterized by elevated production of nitric oxide (NO) [235, 242]. In most murine tumor

models, granulocytic MDSCs are the predominant subset (70-80%) of tumor-associated MDSCs [240, 243].

Lack of a Gr1 analogue in humans has necessitated alternative phenotypic identification of human MDSCs [26], which express common myeloid marker CD33, CD34, CD11b, and IL-4Rα (CD124), but lack expression of the lineage (Lin) markers of DCs and the associated antigen-presentation and co-stimulatory molecules [226, 244]. Human MDSCs are typically defined as $CD33^{+}Lin^{-}HLA\text{-}DR^{-/low}$ or $CD33^{+}CD14^{-}HLA\text{-}DR^{-}$, although recent studies have also demonstrated the presence of additional populations of MDSCs with different phenotypes. These include $CD14^{+}CD11b^{+}HLA\text{-}DR^{low}$ monocytic MDSCs [245-250] and $CD15^{+}$ neutrophil-related immature (i)MDSCs [226], as well as activated granulocytic MDSCs [251, 252] that express high levels of CD66b, CD11b, and VEGFR1 and low levels of CD62L and CD16 [251], Arg1, and/or iNOS [228, 253].

The heterogeneity of MDSCs can be attributed in significant part to the profound plasticity of myeloid cells. This is exemplified by studies examining the adoptive transfer of $CD11b^{+}Gr1^{+}$ cells into congenic mice, which gave rise to immunosuppressive tumor-infiltrating $CD11b^{+}Gr1^{-}F4/80^{+}$ cells [254]. In another study, transfer of $CD11b^{+}Gr1^{+}F4/80^{-}$ cells, isolated from the spleens of EL-4 tumor-bearing congenic mice, into the tumor site of EL-4 tumor-bearing CD57BL/6 recipients, similarly resulted in loss of Gr1 expression (in 70% of the donor cells) and acquisition of F4/80 expression (in 50% of the donor cells), as well as differentiation into $CD11c^{+}$ DCs (in 20% of the donor cells) [255]. *In vitro* culture experiments further substantiated the plasticity of the MDSC phenotype by demonstrating their ability to develop into $CD11b^{+}Gr1^{-}F4/80^{+}$ cells, which retained suppressive capabilities in the absence of cytokines [256, 257]. After transfer of $CD11b^{+}Gr1^{+}CD31^{+}$ MDSCs into normal mice or *in vitro* exposure

to interleukin 4 (IL-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF), MDSCs were also shown to be capable of differentiating into $CD11c^{+}CD86^{hi}$MHC class II^{+} dendritic cells (DCs) [229]. Exposure to IFNγ and TNF could likewise drive MDSC differentiation into functional $CD86^{hi}$MHC class II^{+} antigen-presenting cells (APCs) [258]. These findings demonstrate that depending on the cytokine milieu that prevails during APC maturation, $CD11b^{+}Gr\text{-}1^{+}CD31^{+}$ progenitors can give rise to cells capable of activating $CD8^{+}$ T cells. Along with observations that the differentiation of functional DCs is impaired in tumor-bearing hosts [259], these studies indicate that tumors provide tight microenvironmental control balancing and maintaining the phenotype of local iMCs, DCs, and MDSCs.

1.4.3 MDSC functions

Due to the complex and still-evolving classification of human MDSCs based on phenotypic markers, MDSCs remain most definitively identified by their functional immune-suppressive activity. MDSCs are primarily characterized by their potent ability to suppress both antigen-specific and antigen-non-specific T cell responses [228, 260], with a prominent role for NOS, indoleamine-2,3-dioxygenase 1 (IDO1), and Arg1 activity, as well as the cyclooxygenase 2 (COX2)-driven production of prostaglandin E_2 (PGE_2). MDSCs have also been shown to utilize cell-surface molecules [238, 261-263], TGF-β [245], IL-10 [144, 244], and reactive oxygen species (ROS) [226, 252] to drive immune suppression. T cell suppression by the factors described above has been shown to be mediated through a broad range of mechanisms. Production of reactive nitrogen and oxygen species induce defects in the ζ-chain expression and antigen-recognition capacity of the T cell receptor (TCR) complex [252, 264], as well as defects in the signaling of the IL-2 receptor [265]. Depletion of key amino acids (L-arginine and L-

cysteine) further interferes with both the expression of the TCR complex and the TCR-driven proliferation of antigen-activated T cells [266, 267]. MDSCs have also been shown to disturb proper CD62L-driven migration of naïve T cells to the lymph nodes necessary for priming [268], as well as to interfere with the chemokine-driven localization of effector T cells to intratumoral sites required for efficient tumor rejection [269].

In addition to their function as direct suppressors of effector T cell-mediated immune responses [270, 271], MDSCs further promote tumor progression through the induction, activation, and expansion of regulatory T cells, through both soluble factors and contact-dependent mechanisms [236, 263, 272]. However, their exact role within the network of specific $CD4^+$ T cell subsets, encompassing various populations with distinct cellular and immunological functions (e.g. naïve, effector, regulatory, etc.), remains unclear.

In contrast to MDSC suppression of T cells, current understanding of MDSCs in NK cell suppression is relatively limited, especially in the human tumor context, with conflicting studies in mice suggesting both activation [273] and inhibition [274] of NK cell function through MDSC cell contact involving NKG2D [144, 273], membrane-bound TGF-β1 [144], and NKp30 [248]. The reciprocal effect of activated NK cells on MDSCs is even less well understood, with no studies describing this interaction to date.

1.5 STATEMENT OF THE PROBLEM

It has become clear that the regulation of NK cell, DC, and MDSC activation is complex and under the control of diverse factors and processes, with the character and context of these activation events fundamentally determining the development of significantly different immune

outcomes. It has also become evident that the decisions about the initiation of immune responses to cancer, as well as their character and magnitude, are not determined by individual cells, but are governed by the complex interplay between multiple stimulatory and suppressive cell populations, including NK cells, T cells, DCs, and MDSCs. However, our understanding of the specific interactions that control the initiation, promotion, and suppression of anti-tumor immunity, as well as their underlying regulation, remain incomplete, limiting our ability to design cohesive cancer therapeutic strategies modulating both the induction and suppression of anti-tumor immune responses.

In this work, we investigate the complex cellular interactions driving anti-tumor immunity, focusing on the roles of NK cells. In Chapter 2, we describe distinct functional activities of NK cells in their interaction with DCs, including in the context of late-stage cancer patients, and their unique regulation by distinct cytokines. In Chapters 3 and 4, we describe the NK cell-driven initiation and promotion of DC-induced T cell immunity through critical chemokine regulation of immature DC migration, DC-driven recruitment of naïve T cells to lymph nodes for subsequent T cell priming, and effector T cell infiltration into tumor sites. In Chapter 5, we identify an enriched population of MDSCs within the tumor environment of human ovarian cancer capable of potent $CD8^+$ T cell suppression as well as the nitric oxide-driven skewing of $CD4^+$ T cells toward Th17 immunity. In Chapter 6, we describe an MDSC-mediated mechanism by which activated NK cells and other type-1 lymphocytes promote negative feedback immune suppression, a mechanism enhanced in the human tumor environment for the limitation of anti-cancer responses. Finally, in Chapter 7, we discuss the implications of these findings for a broader understanding of anti-tumor immunity and for future approaches to cancer therapy.

2.0 HELPER ACTIVITY OF NK CELLS DURING THE DC-MEDIATED INDUCTION OF ANTI-TUMOR T CELL IMMUNITY

Adapted from:

Helper activity of natural killer cells during the dendritic cell-mediated induction of melanoma-specific cytotoxic T cells

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2.1 ABSTRACT

NK cells have been shown to mediate important immunoregulatory 'helper' functions in addition to their cytolytic activity. In particular, NK cells are capable of preventing maturation-related DC 'exhaustion', inducing the development of type-1-polarized mature DCs with an enhanced ability to produce IL-12p70, a factor essential for type-1 immunity and effective anti-cancer responses. Here we show that the NK cell-mediated type-1 polarization of DCs can be applied in the context of patients with advanced cancer to enhance the efficacy of DCs in inducing tumor-specific CTLs. In contrast to NK cells activated with IL-2, which mediated efficient DC killing, NK cells isolated from late-stage (stage III and IV) melanoma patients responded with high IFNγ production and the induction of type-1-polarized DCs upon exposure to defined combinations of stimulatory agents, including IFNα plus IL-18. The resulting DCs showed strongly-enhanced IL-12p70 production upon subsequent T cell interaction, compared to immature (i)DCs (average of 19-fold enhancement) and non-polarized IL-1β/TNF-α/IL-6/PGE_2-matured 'standard' (s)DCs (average of 215-fold enhancement). Additional inclusion of poly-I:C during NK-DC co-cultures optimized the expression of CD80, CD86, CD40, and HLA-DR on the resulting $_{NK}$DC1s, increased their CCR7-mediated migratory responsiveness to the lymph node-associated chemokine CCL21, and further enhanced their IL-12-producing capacity. When compared *in vitro* to iDCs and non-polarized sDCs, $_{NK}$DC1s were superior in inducing functional melanoma-specific CTLs capable of recognizing multiple melanoma-associated antigens and killing melanoma cells. These results indicate that the helper function of NK cells can be utilized in clinical settings to improve the effectiveness of DC-based cancer vaccines.

2.2 INTRODUCTION

Dendritic cells (DCs) play a central role in the initiation and regulation of immune responses. They act as carriers of pathogen- and damage-related information, migrating from peripheral sites of pathogen entry and tissue injury to the T cell areas of draining lymph nodes where they prime naïve T cells, providing them with the antigen-specific 'signal 1' and co-stimulatory 'signal 2' [149]. Furthermore, DCs also regulate the balance between the preferential activation of type-1, type-2, and type-17 effector mechanisms of immunity by providing naïve T cells with an additional 'signal 3' [150, 151, 275, 276]. The character of this DC-mediated signal 3 is influenced by the cues provided to them directly by pathogens, by factors produced by injured tissues [275, 277, 278], or by other immune cells capable of sensing transformation or intracellular infections, including natural killer (NK) cells [279, 280].

The argument for the therapeutic use of DCs as cancer vaccines has been recently strengthened by the FDA approval of sipuleucel-T for the treatment of patients with castration-resistant prostate cancer [16]. However, in addition to their ability to deliver antigen, effective DC-based cancer vaccines also need to deliver the co-stimulatory 'signal 2' and IL-12-involving 'signal 3' needed for optimal T cell proliferation and differentiation, respectively [281-283]. Current standard protocols used for the production of 'second-generation' DC vaccines emphasizing these principles yield mature DCs, but with an 'exhausted' ability to produce IL-12p70 [174, 175, 284-286], a crucial factor for the development of type-1 immunity and effective anti-cancer responses [201]. As a result, while standard non-polarized DCs combine a fully mature status (a predictive marker of enhanced immunogenicity [287, 288] with high expression of CCR7, a predictive marker of their lymph node homing capability [289]), they

display only a limited ability to produce IL-12p70 [290-292], ultimately restricting their capacity to induce effective anti-tumor CTL activation.

Several groups, including ours, have previously demonstrated that NK cells can regulate immune responses by activating DCs [189-191] and promoting their differentiation into mature, high IL-12-producing type-1 polarized DCs (DC1s) with an enhanced capacity to induce Th1 and CTL responses [203, 293], the responses most desirable against cancer. These observations, together with a documented role of NK cells during the induction of anti-cancer Th1 and CTL-mediated responses *in vivo* [123, 202, 294-296], suggested the possibility of using NK cells as a tool to generate more effective cancer vaccines. Previously, we reported that NK cells from healthy donors can be activated in a 'two-signal' paradigm to induce DC1 polarization in a mechanism involving IFNγ [203, 297]. The resulting NK-polarized DC1s showed a strongly-elevated capacity to produce IL-12p70 and induce Th1 and CTL responses in polyclonal superantigen-driven models of T cell activation [203, 297].

Here, we report for the first time that this DC1-promoting 'helper' function can be effectively induced in NK cells isolated directly from patients with advanced melanoma under clinically-desirable serum-free conditions, providing a useful tool to induce high numbers of melanoma-specific CTLs capable of recognizing distinct melanoma-associated epitopes and killing melanoma cells.

2.3 MATERIALS AND METHODS

Media and reagents. T cells and tumor cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum and 1% L-glutamine and

Penicillin/Streptomycin (all from Gibco, Invitrogen, Grand Island, NY). IMDM containing 5% human serum (Gemini Bio-Products, West Sacramento, CA) was used as the base medium for the outgrowth of T cell cultures. Two different serum-free medium types were used as the base medium for short-term stimulation of human NK cells as well as to generate DCs: AIM-V medium (Gibco, Invitrogen, Grand Island, NY) and CellGenix DC medium (CellGenix Technologie Transfer GmbH, Freiburg, Germany). The following factors were used throughout the study: granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (Schering-Plough, Kenilworth, NJ); IFNα (Intron A-IFN-α-2b; Schering-Plough); TNFα and IFNγ (both from Miltenyi Biotech, Bergisch Gladbach, Germany); IL-6 (Thermo Scientific, Waltham, MA); PGE_2 (Sigma-Aldrich, St. Louis, MO); IL-18 (MBL International, Woburn, MA); IL-2 (Chiron, Emeryville, CA); IL-7 (PeproTech, Rocky Hill, NJ); and poly-I:C (Sigma-Aldrich, St. Louis, MO). The R24 anti-GD3 monoclonal antibody (mouse IgG3) used in this study was prepared at CellTech (London, UK) and provided by the National Cancer Institute (NCI) BRMP, and was stored at -80°C until use.

NK cell and $CD8^+$ T cell isolation. Peripheral blood from patients with advanced melanoma (stage III and stage IV) and healthy donors was harvested by venipuncture under IRB-approved protocols. NK cells and $CD8^+$ T cells (>95% pure) were isolated by negative magnetic selection using the StemSep system (StemCell Technologies Inc., Vancouver, British Columbia, Canada).

Generation of DCs. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of either healthy donors or melanoma patients (all stage III and IV donors) by density gradient separation using Lymphocyte Separation Medium (Cellgro Mediatech, Herndon, VA). Monocyte fractions were further isolated by CD14 positive selection (Miltenyi Biotech,

Bergisch Gladbach, Germany). Immature DCs were generated from monocytes cultured for 6 days in 24-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at $4x10^5$ cells per well in GM-CSF and IL-4 (both 1,000 IU/ml). To generate 'standard' mature DCs, day 6 immature DCs were cultured for an additional 48 h with IL-1β (10 ng/ml), TNFα (25 ng/ml), IL-6 (1,000 IU/ml), and PGE_2 (10^{-6} mol/L) as previously described [298].

Induction of IFNγ production by NK cells. NK cells were isolated and plated in 96 well plates at $1x10^5$ cells/well. NK cells were stimulated with IFNα (1,000 IU/ml) together with either IL-18 (1 μg/ml), K562 cells ($2x10^4$ cells/well), or melanoma (FEM-X) cells ($1x10^4$ cells/well) in a final adjusted volume of 200 μl. When stated, anti-GD3 antibody (R24) was used to opsonize FEM-X cells. To accomplish this, $1x10^6$ FEM-X cells were placed in 1 ml of tumor culture media and exposed to the R24 antibody at 1 μg/ml for 30 min at room temperature. Cells were then washed three times to remove excess antibody before use.

DC and NK cell co-cultures. Previously isolated and cryopreserved autologous NK cells were thawed and added to DC cultures either directly or separated by Transwell culture inserts (Costar-3413; 0.4μm pore size) at $1.5x10^5$ cells/well to day 6 DC cultures in the presence of IFNα (1,000 IU/ml) and IL-18 (1 μg/ml). When stated, poly-I:C (20 μg/ml) was also added 20 h after co-culture initiation, which was previously determined to be optimal for enhancing its effects.

Flow cytometry. Two and three-colored cell surface immunostaining analyses were performed using a Beckman Coulter Epics XL Flow Cytometer. FITC-labeled anti-human CD86, CD40, and CD3 monoclonal antibodies and the corresponding FITC-isotype (mouse IgG1) control antibodies were purchased from BD Biosciences (San Jose, CA). PE-labeled anti-human CD83 and the corresponding PE-isotype (mouse IgG2b) control monoclonal antibodies were

purchased from BD Biosciences (San Jose, CA). PE-Cy5-labeled anti-human HLA-DR and the corresponding PE-Cy5-isotype (mouse IgG1) control monoclonal antibodies were purchased from Beckman Coulter (Brea, CA). PE-labeled anti-human CCR7 monoclonal antibody was purchased from R&D Systems (Minneapolis, MN) and the corresponding PE-isotype (mouse IgG2a) control antibody was purchased from BD Biosciences (San Jose, CA). PE-labeled MART-1 tetramer (ELAGIGILTV) and the control influenza virus tetramer (GILGFVFTL) were purchased from Beckman Coulter (Brea, CA). Before staining, the cells were treated for 20 min at 4ºC in PBS buffer containing 0.1% NaN_3, 2% human serum, 0.5% BSA, and 1 μg/ml of mouse IgG (Sigma-Aldrich, St. Louis, MO) to block non-specific Fc receptor binding sites. Cells were stained for 40 min at 4ºC followed by washing with PBS buffer containing 0.1% NaN_3 and 0.5% BSA, then fixed and stored in 2% paraformaldehyde until analysis.

DC production of IL-12p70. Dendritic cells were harvested, washed, and plated in 96-well plates at $2x10^4$ cells/well. To mimic the interaction with CD40L-expressing Th cells, CD40L-transfected J558 cells (a gift from Dr. P. Lane, University of Birmingham, United Kingdom), which in previous studies proved equivalent to activated $CD4^+$ T cells and soluble CD40L [285, 299], were added at $5x10^4$ cells/well. Supernatants were collected after 24 h and analyzed by IL-12p70 ELISA (Endogen, Woburn, MA).

Chemotaxis. Dendritic cell migration was induced by CCL21 (6C-Kine-Biosource, Camarillo, CA) and measured using a 96-well 8um pore ChemoTx system (Neuro Probe, Gaithersburgh, MD). $25x10^3$ DC in AIM-V medium were placed on the top of the membrane and permitted to migrate for 90 min at 37°C. Enumeration of migrated DC was determined by counting four random areas in the bottom chamber. Results are expressed as mean DC numbers ± SD of four random areas in duplicate wells.

CTL induction. HLA-A2^{+} melanoma patient-derived CD8^{+} T cells (5x10^{5} cells) were plated in 48-well plates and sensitized by autologous DCs (5x10^{4} cells) that were pulsed with the HLA-A2-restricted peptides MART-1 (26–35), gp100 (209–217), and tyrosinase (368-376). Added to the mix were γ-irradiated (3,000 rad) CD40L-transfected J558 cells (5x10^{4}), which acted as a surrogate for CD40L-expressing CD4^{+} Th cells. At day 4, T cell cultures were supplemented with IL-2 (50 IU/ml) and IL-7 (10 ng/ml). The CD8^{+} T cells were expanded following an additional *in vitro* stimulation (day 12) with irradiated peptide-pulsed autologous PBMCs (1:1 T cell:PBMC ratio). At day 24, the differentially-induced CD8^{+} T cell lines were stimulated with target cells to determine the generated frequency of melanoma-specific CD8^{+} T cells by IFNγ enzyme-linked immunospot (ELISPOT), using either T2 cells (pulsed with the relevant individual antigenic melanoma peptides or the irrelevant HPV-E7 peptide (43-62), or left unpulsed as an additional nonspecific control) or the HLA-A2^{+} and HLA-A2^{-} melanoma cell line targets FEM-X and MEL-397, respectively. The pan-MHC class I blocking antibody (W6/32) was used to determine MHC class I restriction. CTL activity was further assessed by standard 4 h ^{51}Cr-release cytotoxicity assays using the antigen relevant HLA-A2^{+} and irrelevant HLA-A2^{-} melanoma cell lines FEM-X and MEL-397, respectively.

Statistical analysis. Data was analyzed using unpaired and paired t tests (two-tailed) and one-way and two-way ANOVA, where appropriate. Significance was judged at an α of 0.05.

2.4 RESULTS

2.4.1 Differential regulation of human NK cell cytotoxicity and cytokine production by IL-2 and IL-18

While NK cells have been describe to perform diverse lytic and non-lytic functions dependent on prior activation [300], we investigated whether specific NK cell functions may be independently regulated by distinct activating factors. Although NK cell treatment with both IL-2 and IL-18 significantly up-regulated surface expression of CD69 (Fig. 2.1A), a classical marker of NK cell activation, only treatment with IL-2 enhanced NK cell cytotoxicity, including against both Daudi tumor cell targets as well as, importantly, against autologous immature (i)DCs (Fig. 2.1B). In contrast to the potent ability of IL-2-activated NK cells to kill autologous DCs, IL-18-activated NK cells instead demonstrated a preferential ability to respond to another early inflammatory signal, IFNα, with an immune-stimulatory cytokine profile characterized by high expression of IFNγ, TNFα, and GM-CSF, factors known to be essential for DC activation and polarization toward type-1 immune responses critical for effective anti-tumor immunity (Fig. 2.1C).

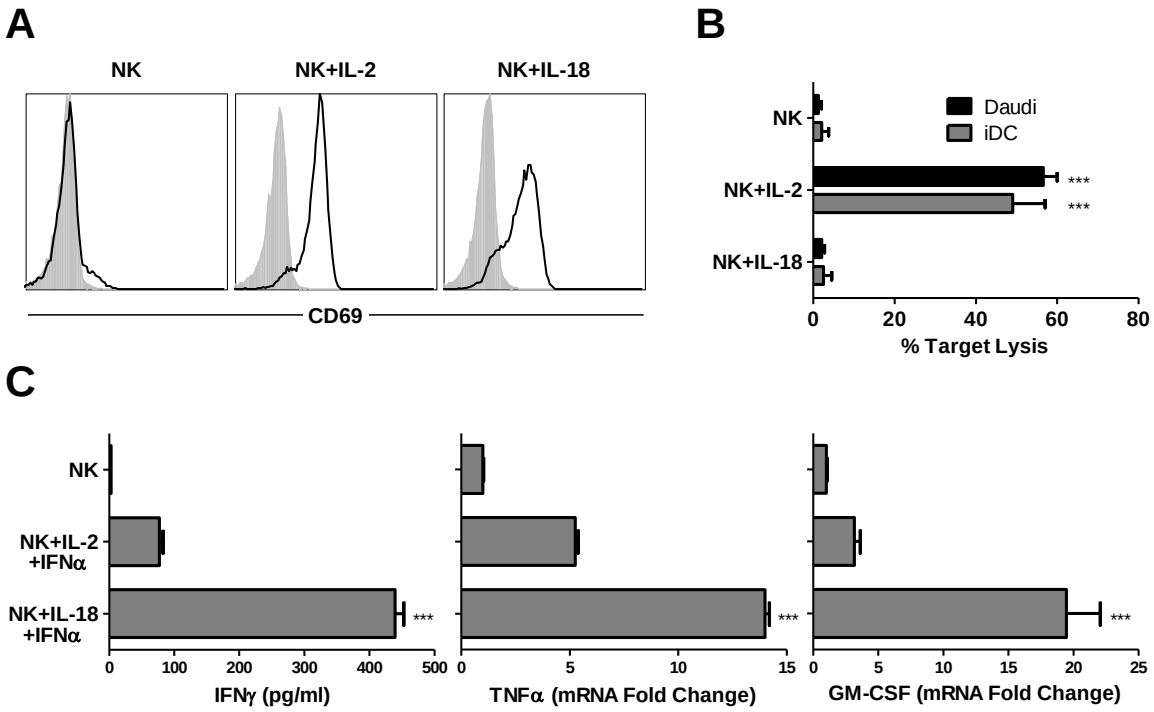

Figure 2.1. IL-2 and IL-18 differentially regulate NK cell cytotoxicity and cytokine secretion.

(A) Representative expression of CD69 on NK cells treated for 24 h with IL-2 or IL-18. (B) Cytotoxic activity of NK cells activated by IL-2 or IL-18 against Daudi tumor cells or autologous immature (i)DCs, as determined by standard 4 h ^{51}Cr-release assay. Data recorded as the mean (± SD) of triplicate cultures. Similar data were obtained in two additional experiments. (C) NK cell expression of IFNγ, TNFα, and GM-CSF measured in 24 h culture supernatants by specific ELISA or 4 h mRNA by RT-PCR. Data recorded as the mean (± SD) of triplicate cultures, and mRNA expression indicated as ratios between the expression of individual chemokine genes and HPRT1. Data represent one of three independent experiments, all yielding similar results. ***$p<0.001$ compared to all groups.

Furthermore, NK cells pre-treated with IL-18 responded synergistically with high IFNγ secretion in response to a diverse range of secondary stimuli in addition to type I interferon, such as exposure to K562 tumor cells or IL-12, IL-2, or IL-15 secondary signals associated with a developing immune response (Fig. 2.2). These results indicate the unique ability of IL-18 to

prime human NK cells toward stable 'helper' immune-stimulatory activity following subsequent NK cell recognition of tumor cells or diverse inflammatory signals.

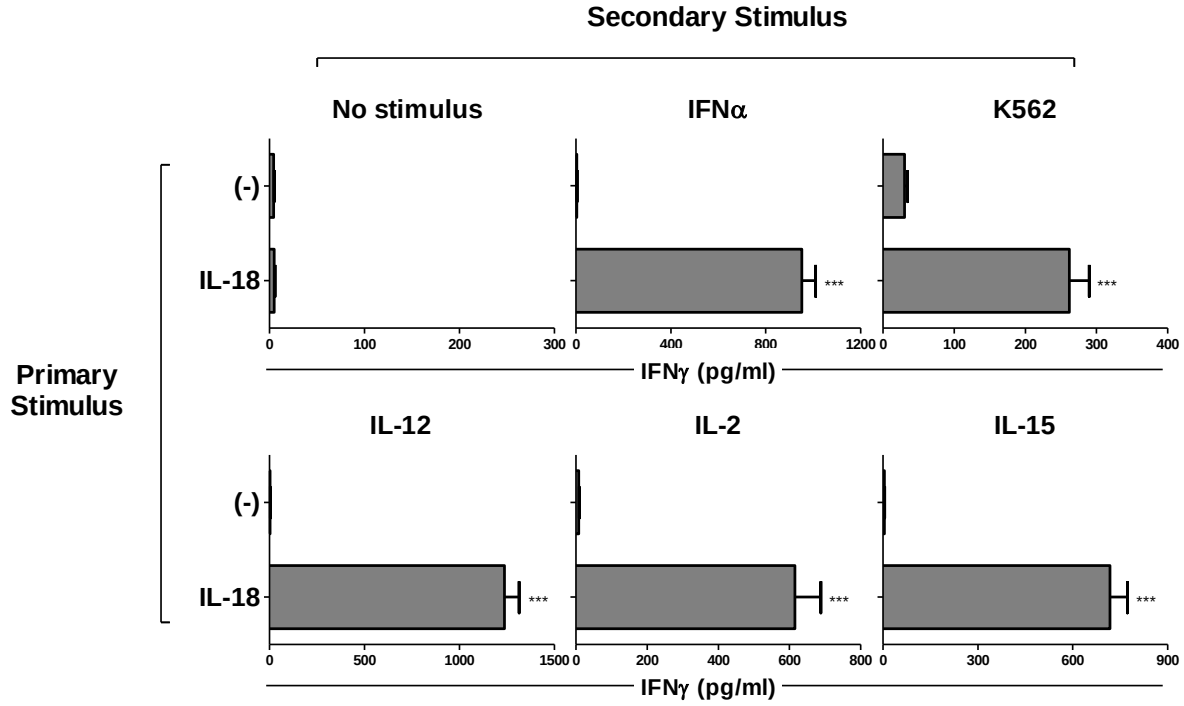

Figure 2.2. IL-18 priming synergizes with multiple secondary stimuli in inducing NK cell secretion of IFNγ.

NK cells were pre-treated for 24 h in the absence or presence of IL-18, washed, and re-plated in the absence or presence of IFNα, K562 tumor cells, IL-12, IL-2, or IL-15. IFNγ in culture supernatants was analyzed after 24 h incubation with the secondary stimulus. Data recorded as the mean (± SD) of triplicate cultures. Data represent one of three independent experiments, which all yielded similar results. ***$p<0.001$ compared to other group.

2.4.2 Intact 'helper' activity of NK cells from melanoma patients: Two-signal activation requirement

In order to test whether NK cells from patients with advanced cancer are similarly functional and whether they respond to the above stimuli in standardized, clinically-desirable serum-free conditions, we first analyzed the cytokine-producing capacity of NK cells derived from late-

stage (stage III and IV) melanoma patients. NK cells were exposed to various activating combinations under serum-free conditions, including IFNα with IL-18, IFNα with the NK cell-sensitive K562 leukemic cell line, or IFNα with the nominally NK cell-insensitive FEM-X melanoma cell line. In accordance with their undisturbed ability to perform helper functions, melanoma patient-derived NK cells produced high levels of IFNγ when stimulated with the combination of IFNα and IL-18, although not when stimulated with either of these factors alone (Fig. 2.3A, top). Similarly, the combination of IFNα with NK cell-sensitive K562 cells or with opsonized NK cell-insensitive FEM-X melanoma tumor cells, but not with any of these individual stimuli, effectively induced freshly-isolated NK cells from melanoma patients to secrete IFNγ (Fig. 2.3A, middle and bottom).

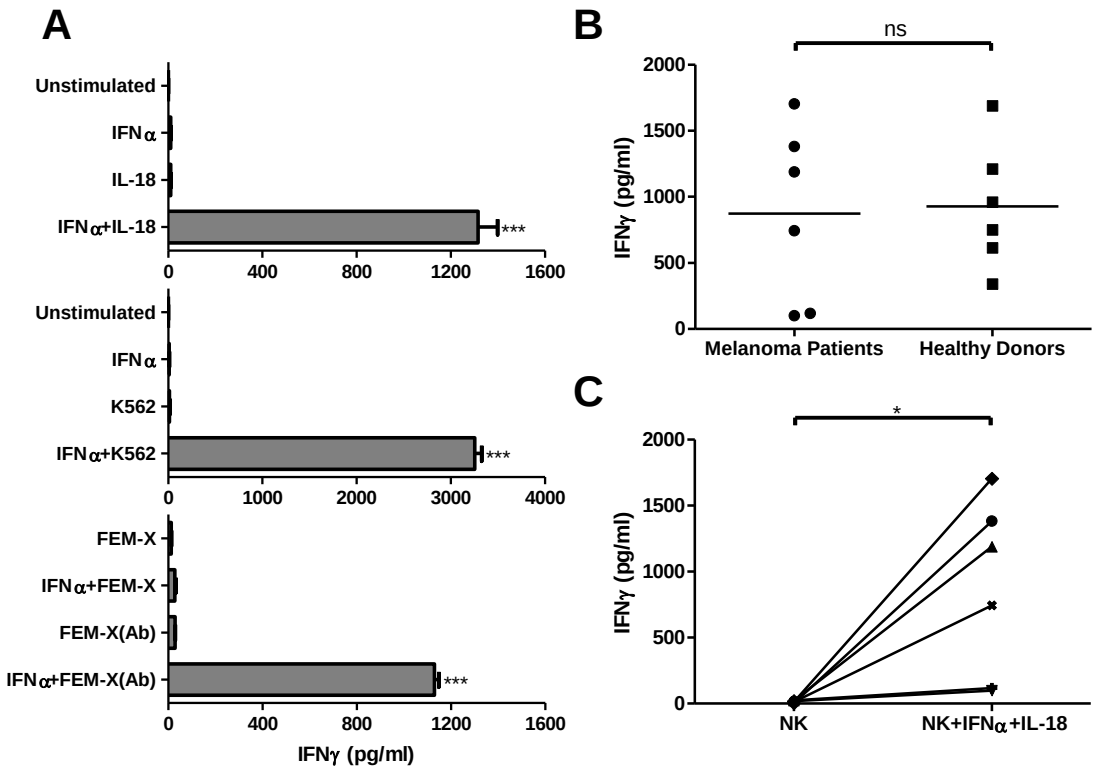

Figure 2.3. Two-signal activation requirement for IFNγ production by NK cells isolated from late-stage melanoma patients.

Negatively-isolated NK cells were incubated for 24 h in the presence of the indicated combinations of activating factors. Supernatants were subsequently assayed by ELISA for the presence of IFNγ. (A) NK cell production of IFNγ in response to stimulation with IFNα and/or IL-18 (top); IFNα and/or exposure to NK cell-sensitive K562 leukemia tumor cells (middle); or IFNα and/or exposure to antibody (R24)-opsonized, nominally NK cell-resistant FEM-X melanoma cells (bottom). Data shown represents one of six independent experiments, which all yielded similar results. Data recorded as the mean (± SD) of triplicate cultures. (B) Comparison of IFNγ production by NK cells derived from six melanoma patients or six healthy donors in response to stimulation with IFNα and IL-18. Data recorded as the mean of triplicate cultures for each patient or healthy donor. (C) Comparison of IFNγ production by unstimulated or IFNα/IL-18-stimulated NK cells isolated from individual melanoma patients. Data is presented as the mean of triplicate cultures for each patient (total of 6 patients). ***$p<0.001$, *$p<0.05$, ns: $p>0.05$ compared to indicated groups or compared to all groups when not specifically indicated.

While immune cells from tumor-bearing individuals are known to display multiple functional defects [301], the ability of NK cells to respond to two-signal stimulation was similar when comparing healthy donors and melanoma patients, although a significant variation in the absolute levels of IFNγ production was observed in both groups of donors (Fig. 2.3B). Despite this variability, all patients demonstrated strong increases in IFNγ secretion following activation (Fig. 2.3C), suggesting intact NK helper function even in patients with late-stage cancer.

2.4.3 NK cells from melanoma patients prime DCs for an enhanced ability to produce IL-12p70

Having established that melanoma patients' NK cells are competent in their ability to respond to two-signal stimulation with high IFNγ production, we tested if these two-signal-activated NK cells could also promote the development of autologous type-1-polarized DCs (DC1s) with an elevated, rather than 'exhausted' [284, 285], ability to produce IL-12p70. To accomplish this,

cryopreserved autologous NK cells from late-stage melanoma patients were thawed and added to day 6 immature DCs for 48 h in the presence of IL-18 and IFNα.

As shown in Figure 2.4, while DCs matured with the "standard" cytokine cocktail of IL-1β/TNF-α/IL-6/PGE_2 (sDCs), a vaccine protocol used extensively in recent clinical trials [298, 302], showed a diminished capacity to produce IL-12p70 (compared to immature (i)DCs from the same donors), the DCs induced by two-signal-activated NK cells produced greatly enhanced levels of IL-12p70 (Fig. 2.4A). Control DCs exposed to the mix of NK cell-activating factors (IL-18 and IFNα) in the absence of NK cells failed to produce elevated levels of IL-12p70, demonstrating that NK cells are themselves critical, rather than solely IL-18 and IFNα, for this enhancement of IL-12 production by DCs.

We performed transwell experiments to address whether cell-to-cell contact played a role in this NK cell-induced enhancement of IL-12p70 expression. In accordance with the previously-demonstrated key role of the soluble factor IFNγ in NK cell-mediated DC polarization [203], two-signal-activated NK cells could enhance the IL-12p70-producing capacity of bystander DCs independent of cell-to-cell contact, although the IL-12-enhancing effects were maximal in the presence of cell contact (Fig. 2.4B).

Consistent with the notion that the ability of NK cells to perform 'helper' functions is preserved even in patients with advanced cancer, similar results could be consistently obtained with blood from different patients with stage III-IV melanoma (Fig. 2.4C). On average, the NK cell-induced DC1s demonstrated over 200-fold greater capacity to produce IL-12p70 compared to sDCs generated from the same individual patient, and over 19-fold greater capacity compared to immature DCs from the same patient. This degree of enhancement was comparable to our observations from healthy donors [203, 297]. Such enhanced ability to produce IL-12 was

preserved for at least 24 h after harvesting of the DCs (Fig. 2.4D), suggesting that the function of these NK cell-induced DC1s will remain intact following their therapeutic application and migration to draining lymph nodes in clinical settings.

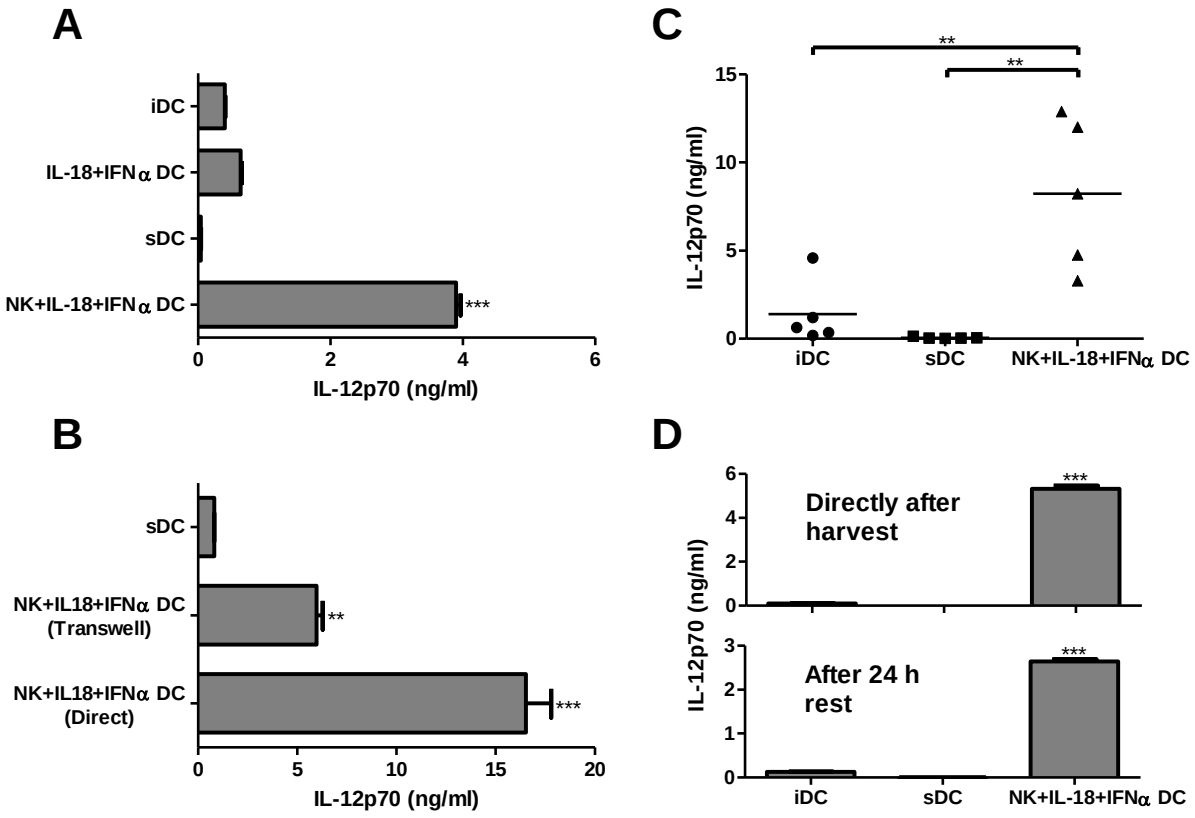

Figure 2.4. Two-signal-activated NK cells from late-stage melanoma patients stably induce DCs with an enhanced capacity to produce IL-12p70.

Previously isolated and cyropreserved NK cells were added to autologous day 6 DCs (2-3x10^5 cells/well) in the presence of IFNα and IL-18. After 48 h, the DCs were harvested, plated, and exposed to J558-CD40L to induce IL-12p70 production. IL-12p70 concentrations in 24 h supernatants were determined by ELISA. (A) IL-12p70 production by untreated immature DCs (iDCs), DCs treated with the standard cytokine maturation cocktail of TNFα/IL-1β/IL-6/PGE_2 (sDCs), or DCs treated with IL-18/IFNα with or without autologous NK cells. Data recorded as the mean (± SD) of triplicate cultures. Data shown was obtained from one representative experiment of five performed, all yielding similar results. (B) IL-12p70 production by DCs treated with the standard cytokine cocktail (sDCs) or autologous NK cells with IL-18/IFNα in direct or transwell-separated co-cultures. Data recorded

as the mean (± SD) of triplicate cultures. Data from one representative experiment of two performed, both of which yielded similar results. (C) IL-12p70 production by untreated immature DCs (iDCs), DCs treated with the standard cytokine maturation cocktail (sDCs), or DCs treated with autologous NK cells and IL-18/IFNα. Data recorded as the mean of triplicate cultures for each patient (total of 5 patients). (D) IL-12p70 production by differentially-matured DCs stimulated with CD40L directly after harvesting (top) or after an additional 24 h of culture in the absence of maturation factors (bottom). Data presented as the mean (± SD) of triplicate cultures for each patient. Data from one representative experiment of three performed, all of which yielded similar results. ***$p<0.001$, **$p<0.01$ compared to indicated groups or compared to all groups when not specifically indicated.

2.4.4 $_{NK}$DC1s express high levels of maturation-associated co-stimulatory, antigen presentation, and lymph node migratory molecules: Stability of the $_{NK}$DC1 phenotype

The effective induction of primary T cell responses during vaccination requires the action of fully mature DCs that express high levels of co-stimulatory and antigen presentation molecules, and that are capable of migrating to lymph nodes in response to CCR7 ligands [289]. While the production of IL-12p70 is indeed critical to their ability to induce tumor-specific Th1 cells and CTLs [290-292], the IL-12-producing capacity of DCs often inversely correlates with the maturation status of the DC [285, 299]. Therefore, we examined the maturation status of the DCs in our NK-DC co-culture system by surface flow cytometric analysis. As shown in Figure 2.5, the DCs co-cultured with autologous NK cells in the presence of IL-18 and IFNα demonstrated a partially-activated phenotype, manifested by enhanced expression of the co-stimulatory molecules CD80, CD86, and CD40, compared to immature DCs. However, additional co-stimulation with a TLR3/RIG-I/MDA5-ligand, polyinosinic:polycytidylic acid (poly-I:C), was needed to optimize expression of these molecules to levels comparable to the sDC maturation cocktail.

Similar to their IL-12-producing capacity, the mature surface phenotype of $_{NK}$DC1s was maintained after 24 h of additional culture in the absence of maturation factors (Fig. 2.5, bottom). In all cases, the presence of NK cells in the maturation cultures was critical in inducing optimal DC maturation, compared to the IL-18/IFNα-exposed- or IL-18/IFNα/poly-I:C-exposed DCs (without NK cells) from the same patients (*data not shown*).

Besides enhanced expression of co-stimulatory factors and the ability to produce high levels of IL-12p70, the capacity of DCs to induce immune responses and serve as effective cancer vaccines is also influenced by their ability to migrate in response to lymph node-produced chemokines, dependent on DC expression of CCR7 [283, 303]. Similar to maturation-associated co-stimulatory molecules, CCR7 surface expression was enhanced by DC exposure to two-signal-activated NK cells, especially with the additional presence of poly-I:C (Fig. 2.5, bottom). In agreement with prior reports [289], this enhanced expression of CCR7 was found to be functional in terms of migratory responsiveness to CCL21, a lymph node-associated chemokine ligand for CCR7, and was greatly augmented by direct NK-DC cell contact in transwell experiments (Appendix Fig. 1). Moreover, CCR7 expression on $_{NK}$DC1s was further modestly increased after 24 h of additional culture in fresh media (Fig. 2.5, bottom), consistent with our recent report describing the CCR7 regulation in type-1-polarized DCs induced by soluble NK cell-related factors [304].

Similar to the enhanced expression of surface molecules involved in T cell stimulation and lymph node-homing, the presence of poly-I:C in the IL-18/IFNα-activated NK-DC co-cultures further augmented the IL-12p70-producing capacity of DCs (Fig. 2.5, right), making such conditions preferable for our prospective applications. While poly-I:C stimulation alone can result in the augmentation of IL-12-production by maturing DCs [305], the high capacity for IL-

12-production observed in $_{NK}$DC1s could not be stably imprinted by the combination of IL-18, IFNα, and poly-I:C in the absence of NK cells (Appendix Fig. 2), demonstrating the key role for NK cells.

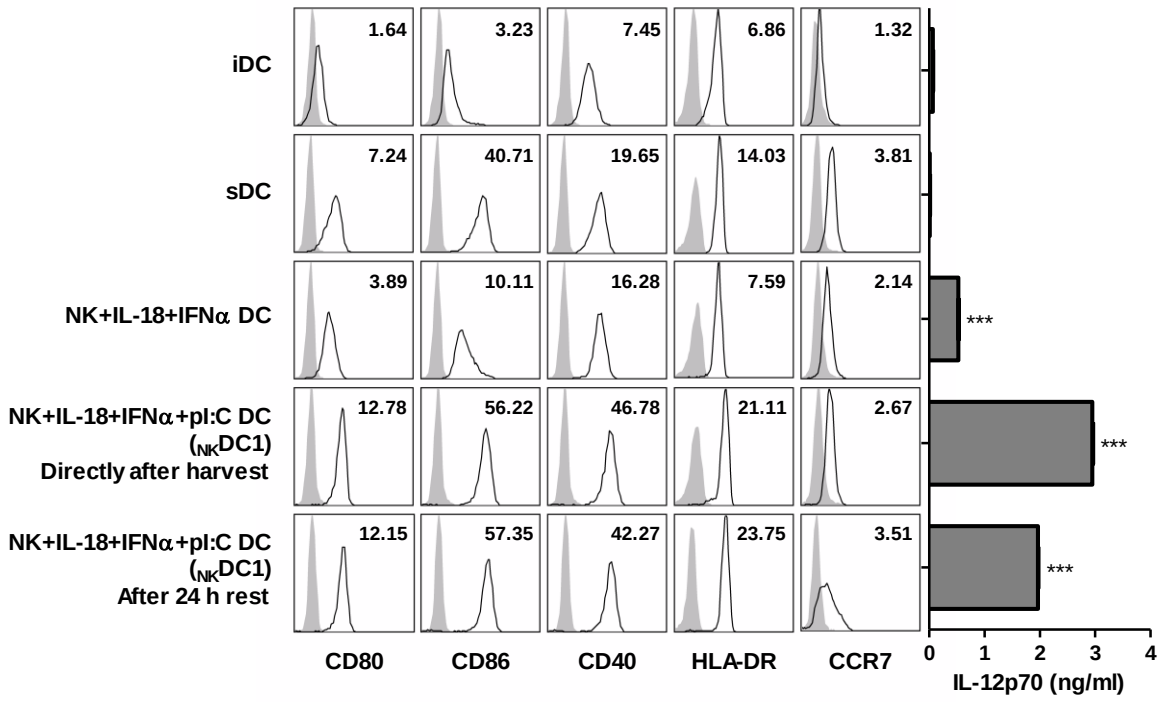

Figure 2.5. Inclusion of poly-I:C in NK-DC co-cultures results in $_{NK}$DC1s with optimal surface expression of T cell-activating molecules and CCR7 and optimal ability to produce IL-12p70.

Surface expression (open histograms) of CD80, CD86, CD40, HLA-DR, and CCR7 on untreated immature DCs (iDCs), DCs treated with the standard cytokine maturation cocktail (sDCs), or DCs treated with autologous NK cells and IL-18/IFNα with or without poly-I:C. Surface expression was analyzed directly after DC harvesting or after an additional 24 h of culture in the absence of maturation factors. Gray histograms represent isotype controls. Inset numbers represent fold MFI increase over isotype controls. *Right:* The corresponding IL-12p70 production after J558-CD40L-stimulation. Data from one representative experiment of three performed, all of which yielded similar results. ***p<0.001 for $_{NK}$DC1s (NK/IL-18/IFNα/poly-I:C DCs) compared to iDCs, sDCs, and NK/IL-18/IFNα DCs, or for NK/IL-18/IFNα DCs compared to iDCs and sDCs.

2.4.5 $_{NK}$DC1s induce high numbers of tumor-specific CTLs capable of recognizing multiple melanoma antigens and killing melanoma cells

To determine the relative ability of $_{NK}$DC1s to induce tumor-specific CTL responses, $_{NK}$DC1s generated from HLA-A2^{+} melanoma patients (stage III and IV) were loaded with HLA-A2-restricted melanoma-associated antigenic peptides and used to sensitize autologous blood-isolated CD8^{+} T cells *in vitro*. Parallel control cultures included iDCs or sDCs, in order to compare $_{NK}$DC1s to, respectively, immature/partially-mature DCs used in the FDA-approved prostate vaccine [306], or to fully-mature DCs extensively tested in past clinical trials [298, 302]. DCs exposed to IL-18/IFNα/poly-I:C in the absence of NK cells were also used as additional controls. Following two rounds of *in vitro* sensitization, the expanded CD8^{+} T cells were harvested and used as responders against HLA-A2^{+} T2 cells pulsed with individual peptides, or against HLA-A2^{+} melanoma cells (FEM-X) or control HLA-A2^{-} melanoma cells (MEL-397).

As shown in Figures 2.6A and 2.6B, $_{NK}$DC1s proved to be superior in generating high numbers of functional melanoma-specific CTLs, as determined by IFNγ ELISPOT against distinct MART-1, gp100, and tyrosinase epitopes, and by tetramer staining of MART-1-specific T cell receptors. This enhanced CTL-inducing activity of $_{NK}$DC1s was strictly dependent on the presence of NK cells, and was not observed in the DCs activated by IL-18/IFNα/poly-I:C alone (Appendix Fig. 3). Importantly, $_{NK}$DC1-sensitized CD8^{+} T cell cultures contained a larger percentage of CTLs not only capable of specifically recognizing peptide-loaded T2 cells, but also capable of specifically detecting and killing HLA-A2^{+} melanoma cells (Fig. 2.6, C and D). This demonstrates that $_{NK}$DC1-sensitized CD8^{+} T cells are able to detect physiologic amounts of tumor-associated antigens and are capable of killing actual tumor cells, which often show enhanced resistance to immune elimination [307-309].

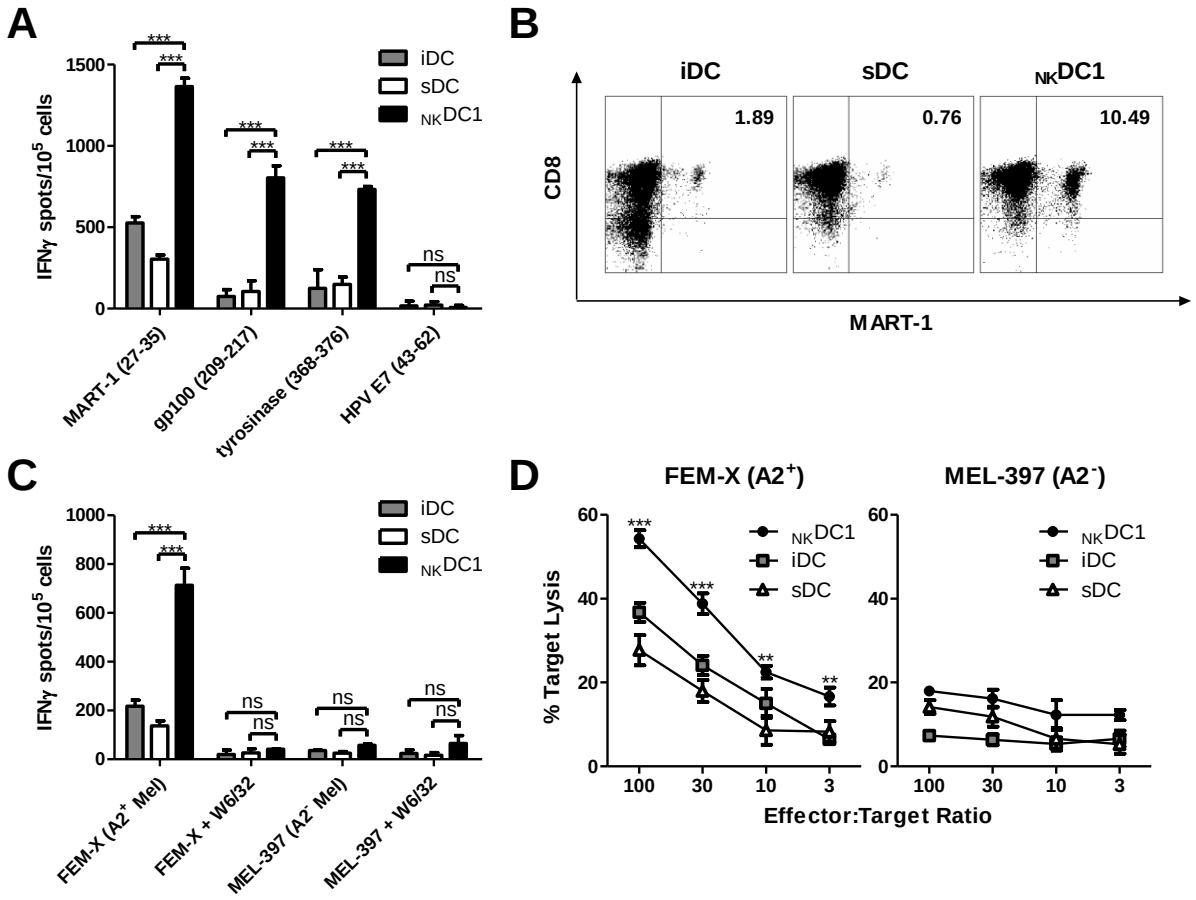

Figure 2.6. $_{NK}$DC1s are efficient inducers of melanoma-specific CTLs.

Immature (i)DCs, sDCs, and $_{NK}$DC1s from HLA-A2$^+$ stage III and stage IV melanoma patients were pulsed with MHC Class I-restricted melanoma-associated peptides and used to sensitize autologous CD8$^+$ T cells. CTLs were assayed on day 24 of culture. (A) Frequencies of IFNγ-producing CD8$^+$ T cells responsive to T2 cells loaded with individual peptides, as determined by ELISPOT assay. Data recorded as the mean (± SD) of triplicate cultures. Data shown is from one representative experiment of three performed. (B) Flow cytometric analysis showing percentage of tetramer-positive MART-1-specific CD8$^+$ T cells generated through *in vitro* stimulation with melanoma peptide-pulsed, differentially-activated DCs. Inset numbers represent percentages of CD8$^+$ MART-1$^+$ cells. Results from one representative experiment of three performed. (C) Frequencies of IFNγ-producing CD8$^+$ T cells responsive to the relevant (HLA-A2$^+$) and irrelevant (HLA-A2$^-$) target melanoma cell lines FEM-X and MEL-397, respectively, as determined by ELISPOT assay. Blockade with the W6/32 pan-MHC Class I-neutralizing antibody was used to demonstrate the MHC Class I-dependence of the T cell recognition. Data recorded as the mean (± SD) of triplicate

cultures. Data shown is from one representative experiment of three performed. (D) Antigen-specific cytotoxic activity of CTLs induced by $_{NK}$DC1s, iDCs, or sDCs against FEM-X (HLA-A2^{+}) and MEL-397 (HLA-A2^{-}) melanoma cell lines, as determined by standard 4 h ^{51}Cr-release assay. Data recorded as the mean (± SD) of triplicate cultures. Similar data were obtained in two additional experiments. ***p<0.001, **p<0.01, *ns: p*>0.05 compared to indicated groups or compared to all groups when not specifically indicated.

2.5 DISCUSSION

Numerous preclinical studies and clinical trials have individually employed either NK cells or DCs as tools in the immunotherapy of cancer. While the results of animal studies, as well as observations of clinical responses in individual cancer patients, have shown the potential benefits of such NK- and DC-based cancer therapies, their overall clinical efficacy has been disappointing [310-312]. The current results and recent demonstrations that NK cells can play a critical immunoregulatory "helper" role to support the induction of Th1 and CTL-mediated responses in mouse models [123, 202, 294-296] and human *in vitro* studies [203, 297] suggest the potential for improving the effectiveness of DC-based anti-tumor vaccination strategies by exploiting the interactions between NK cells and antigen-carrying DCs.

We previously showed that type-I IFNs and tumor-associated activation ligands expressed on NK cell-sensitive K562 cells can synergistically induce the NK cell-mediated polarization of DC1s in healthy donors, in a mechanism dependent on IFNγ production. The current study, showing consistent generation in serum-free conditions of functional $_{NK}$DC1s from the blood of different patients with advanced (stage III and IV) melanoma, demonstrates the potential for translating these findings into clinically-relevant settings using NK cells and DCs isolated directly from cancer patients. The ability of IL-18 to act in synergy with IFNα as a

substitute for tumor lines provides a user-friendly, highly-reproducible, and potentially safer method of harnessing the DC1-polarizing activity of NK cells. The added positive effect of poly-I:C on the IL-12-producing function of $_{NK}$DC1s and their expression of maturation-associated co-stimulatory and lymph node-homing molecules is consistent with its ability to enhance the cross-talk between NK cells and DCs recently observed in human *in vitro* [90, 313] and mouse *in vivo* [314-316] settings.

In addition to promoting effective NK-DC interactions (and the resulting type-1 polarization of DCs) *ex vivo* during the generation of cell-based vaccines, the two-signal activation paradigm required for NK cell helper activity provides a rationale for *in vivo* approaches involving co-delivery of such cytokines as IL-18 and IFNα, or the combination of IFNα and tumor-specific opsonizing antibodies. Such therapies are likely to be particularly effective when further combined with adoptive transfer of *ex vivo* expanded/activated NK cells. In the case of antibody-utilizing therapies, in addition to the IgG1- or IgG3-antibody-triggered activation of CD16 on NK cells and resulting cytokine production [317, 318] and DC1-polarization [203], antibody-directed NK cell-mediated lysis of nominally NK cell-resistant tumors may also provide potential antigen for cross-presentation by bystander DCs, further enhancing active immunization.

A number of questions still remain concerning the potential differential impact of distinct combinations of NK cell-activating factors on NK cells and their ability to modulate DC function. It has been shown that NK cells, in analogy to Th cell differentiation, can also differentiate into polarized subsets displaying different cytokine patterns, producing a wide variety of factors including IFNγ, TNFα, IL-4, IL-5, IL-13, and IL-10 with both immune-stimulatory and immune-suppressive functions [319]. It is therefore conceivable that depending

on the mode of their activation, instead of promoting DC-mediated type-1 immunity, NK cells activated by a particular stimulus may instead drive a type-2 response [320, 321], or even suppress DC function altogether [322], thus highlighting the need for the careful selection of NK cell-activating signals to be used in clinical settings.

The current data demonstrate the feasibility and rationale for the clinical application of immunotherapies of melanoma and other cancers utilizing the positive feedback between NK cells and DCs. The high activity of antigen-loaded $_{NK}$DC1s in inducing tumor-specific CTLs makes them interesting candidates for clinical evaluation as cancer vaccines as an alternative to standard DCs or type-1-polarized DCs induced in less physiologic conditions, using the combination of NK cell-related soluble factors [175]. While NK cell-derived IFNγ appears to be the obligatory polarizing component in the development of $_{NK}$DC1s [174, 203], additional factors may also likely be involved, as indicated in our transwell experiments demonstrating enhanced DC function when direct contact between the two cell types was permitted. This latter effect may indicate the involvement of additional membrane-bound molecules, as observed in related systems [128, 189-191], but may also reflect the close proximity of the two cell types and higher concentrations of soluble factors. The potential contribution of additional NK cell-related factors to the helper activity of NK cells and the phenomena of DC activation is a subject of our current analyses.

3.0 HELPER NK CELLS ATTRACT AND COLLABORATE WITH DCS TO PROMOTE THE RECRUITMENT OF CD8$^+$ T CELLS TO THE CANCER ENVIRONMENT

Adapted from:

IL-18-primed helper NK cells collaborate with dendritic cells to promote recruitment of effector CD8$^+$ T cells to the tumor microenvironment

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3.1 ABSTRACT

The chemokine-driven interactions of immune cells are essential for effective anti-tumor immunity. Human natural killer (NK) cells can be primed by IL-18 for unique 'helper' activity, promoting dendritic cell (DC) activation and DC-mediated induction of type-1 immune responses against cancer. Here we show that such IL-18-primed 'helper' NK cells produce high levels of the immature DC (iDC)-attracting chemokines CCL3 and CCL4 upon exposure to tumor cells or the additional inflammatory signals IFNα, IL-15, IL-12, or IL-2. These 'helper' NK cells potently attract iDCs in a CCR5-dependent mechanism and induce high DC production of CXCR3 and CCR5 ligands (CXCL9, CXCL10, and CCL5), facilitating the subsequent recruitment of type-1 effector $CD8^+$ T (T_{eff}) cells. Using cells isolated from the malignant ascites of patients with advanced ovarian cancer, we demonstrate that 'helper' NK cell-inducing factors can be used to enhance the local production of T_{eff} cell-recruiting chemokines. This study demonstrates for the first time the unique chemokine expression profile of 'helper' NK cells, and highlights the potential for utilizing two-signal-activated NK cells to promote homing of type-1 immune effectors to the human cancer environment.

3.2 INTRODUCTION

Natural killer (NK) cells are innate sentinel cells recognizing early signs of tissue stress, infection, or transformation [200, 300]. NK cells integrate signals from activating and inhibitory receptors engaged by pathogen products and/or products released from affected cells, such as type I interferons (IFNα/β) [89, 323], and play a critical 'helper' role in initiating and directing

dendritic cell (DC)-regulated immune responses [189-191, 202]. Constituting an early source of IFNγ and TNFα, NK cells are capable of promoting DC maturation and DC-mediated induction of type-1-polarized helper $CD4^+$ T cell (Th1) and cytotoxic $CD8^+$ T cell (CTL) responses [128, 153, 203, 297].

Resting NK cells require activation for the acquisition of different effector functions, and specific NK cell functions can be preferentially driven by distinct cytokines, including IL-18 [200]. IL-18 is an IL-1 family cytokine widely expressed by multiple barrier cell types, including epithelial cells in the gut and lung and keratinocytes in the skin, and by early-responding innate cells, such as monocytes and macrophages [324]. Expression of the IL-18 pro-cytokine is further enhanced by toll-like receptor signaling, with production of the mature cytokine controlled by activated caspase-1 [325]. Caspase-1 activity in turn depends on inflammasome activation, which is likewise downstream of pattern receptor recognition [325]. Thus, IL-18 represents an early product of the developing response to tissue damage, infection, or transformation. We have previously shown that unlike IL-2, which promotes 'killer/effector' NK cell differentiation characterized by enhanced cytotoxicity against tumor and DC targets, IL-18 uniquely primes human NK cells for preferential non-cytotoxic 'helper' activity upon subsequent stimulation with multiple distinct secondary factors, including tumor cells and type I interferons. We demonstrated that these IL-18-primed 'helper' NK cells are capable of inducing DC activation and potentiating DC-mediated induction of tumor-specific Th1 and CTL adaptive immune responses through an IFNγ- and TNFα-dependent mechanism [203, 297], including in patients with late-stage cancer [326]. Here, we investigate whether IL-18 may also uniquely regulate human NK cell chemokine production to enhance interaction with DCs, and subsequently

influence productive chemokine-driven interactions with effector T cells, particularly in the context of the human tumor environment.

While prior studies have described the ability of activated DCs to attract NK cells, in mechanisms involving CXCR3 and CXCR1 and their chemokine ligands [123, 327, 328], our current data indicate that human NK cells can initiate chemokine-driven NK-DC interaction in response to signals associated with infection or neoplastic cell transformation. We show that IL-18-primed NK cells can act as the inducers of local immune cell accumulation, promoting the CCR5-dependent attraction of immature DCs and driving subsequent DC production of the effector $CD8^+$ T (T_{eff}) cell-recruiting chemokines CXCL9, CXCL10, and CCL5, both in cells isolated from the blood of healthy donors as well as in tumor-associated cells isolated from the malignant ascites of advanced (stage III-IV) ovarian cancer (OvCa) patients.

3.3 MATERIALS AND METHODS

Media, cell lines, and reagents. Serum-free CellGenix DC medium (CellGenix Technologie Transfer GmbH) was used for short-term culture of human NK cells and for DC generation. T cells, ovarian cancer ascites-derived cells, and K562 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum and 1% L-glutamine and penicillin/streptomycin (all from Gibco, Invitrogen). K562 cells were obtained from American Type Culture Collection, expanded and cryopreserved after receipt, and used for experiments from recently thawed stocks. The following factors were used throughout the study: IL-18 (MBL International); IL-2 (Chiron); IFNα (Intron A, IFN-α-2b; Schering-Plough); IL-12 (PeproTech);

IL-15 (Sigma-Aldrich); IL-1β (Miltenyi Biotech); and granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (Schering-Plough).

NK cell and CD8$^+$ T cell isolation. Peripheral blood from healthy donors was harvested by venipuncture under IRB-approved protocols. NK cells ($CD56^+CD3^-$) and naïve $CD8^+$ T cells ($CD8^+CD45RA^+CCR7^{high}CD45RO^-CD56^-CD57^-$) were isolated by negative magnetic selection (>95% pure in both cases) using the EasySep system (StemCell Technologies), according to the manufacturer's protocol. When indicated, $CD3^-CD56^{bright}CD16^-$ and $CD3^-CD56^{dim}CD16^+$ NK cell subsets were flow-sorted using a MoFlo high-speed cell sorter (DakoCytomation), after labeling with appropriate antibodies.

Blood DC isolation. Human blood DCs, including all three major subsets [$CD1c^+$ (BDCA-1^+), $CD141^+$ (BDCA-3^+), and $CD304^+$ (BDCA-4^+)], were isolated from healthy donor peripheral blood by magnetic selection using the Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer's protocol. Cells were >95% HLA-DR^+ and >99% $CD14^-CD19^-CD3^-CD56^-$.

Generation of DCs. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy donors by density gradient separation using Lymphocyte Separation Medium (Cellgro Mediatech). Monocyte fractions were further isolated by CD14 positive magnetic selection (Miltenyi Biotech). Immature DCs were generated from monocytes cultured for 6 days in 24-well plates at $4x10^5$ cells/well in GM-CSF and IL-4 (both 1000 IU/ml).

NK cell stimulation and DC co-culture. NK cells were isolated and plated in 48-well plates at $1x10^6$ cells/ml. NK cells were stimulated with IL-18 (200 ng/ml) or IL-2 (250 IU/ml) together with IFNα (1000 IU/ml), IL-12 (5 ng/ml), IL-2 (250 IU/ml), IL-15 (100 ng/ml), or K562 cells ($1x10^5$ cells/well). Alternatively, NK cells were pre-treated with IL-18 or IL-2 for 24 h,

washed thoroughly, and re-plated in the presence of IFNα, IL-12, IL-2, IL-15, or K562 cells as a secondary stimulus. Expression of chemokines was analyzed at 4 h by quantitative real-time PCR or at 24 h by specific ELISA. When indicated, NK cells were pre-treated for 30 min at 37°C with blocking antibodies to NKG2D (clone 1D11; 10 μg/ml; Biolegend), NKp30 (clone P3015; 10 μg/ml; Biolegend), or DNAM-1 (clone DX11; 10 μg/ml; Abcam) before co-culture with K562 cells. For NK cell activation of DCs, previously isolated and cryopreserved autologous NK cells were thawed and added to DC cultures at $1.5x10^5$ cells/well to day 6 DC cultures in the presence of IL-18 (200 ng/ml) and IFNα (1000 IU/ml). When indicated, soluble decoy receptors to TNFα (sTNFR1; 1 μg/ml; R&D Systems) and IFNγ (sIFNγR1; 10 μg/ml; R&D Systems) were added to cultures at co-culture initiation. Supernatants were collected at 48 h for chemokine analysis. To assess the stability of DC chemokine production, NK-DC co-cultures were harvested and washed, and NK cells were removed by CD56 positive magnetic selection (StemCell Technologies). DCs were then re-plated in 96-well plates at $2x10^4$ cells/well. To mimic interaction with CD40L-expressing $CD4^+$ T cells, DCs were co-cultured with CD40L-transfected J558 cells (a gift from Dr. P. Lane, University of Birmingham, United Kingdom) at $5x10^4$ cells/well, which in previous studies proved equivalent to activated $CD4^+$ T cells and soluble CD40L [175]. Supernatants were collected after 24 h and analyzed by specific ELISAs for CXCL9, CXCL10, CCL5, and CCL22 (PeproTech).

Generation of effector $CD8^+$ T cells by *in vitro* sensitization. Naive $CD8^+$ T cells were activated with staphylococcal enterotoxin B-pulsed DCs matured from day 6 immature DCs by 36 h treatment with TNFα (50 ng/ml), IL-1β (25 ng/ml), IFNγ (1000 IU/ml), poly-I:C (20 μg/ml), and IFNα (3000 IU/ml), as previously described [175]. DCs matured in this manner have been extensively demonstrated to be efficient inducers of $CD45RO^+granzymeB^{high}$ effector-type

$CD8^+$ T cells (T_{eff} cells) expressing high levels of the peripheral homing chemokine receptors CXCR3 and CCR5 [175, 329]. On days 5–6, expanded $CD8^+$ T cells were analyzed to confirm CTL phenotype and expression of chemokine receptors, and were subsequently used for chemotaxis assays.

Chemotaxis. Chemotaxis assays were performed using 24-(Trans)well plates with 5 μm pore size polycarbonate membranes (Corning), as previously described [173]. For DC chemotaxis, the lower chamber was filled with supernatants from 36 h cultures of NK cells treated with IL-18 (200 ng/ml) or IL-2 (250 IU/ml) together with IFNα (1000 IU/ml) in CellGenix medium, and the upper chamber was loaded with blood-isolated DCs or day 6 monocyte-derived immature DCs ($2x10^5$). When indicated, DCs were treated for 30 min with an anti-CCR5 blocking antibody (Clone 2D7, 20 μg/ml; BD Biosciences) before chemotaxis to block CCR5-dependent chemotaxis. Alternatively, DCs were treated for 30 min with recombinant CCL3, CXCL8, XCL1, CCL20, or CXCL12 (all at 200 ng/ml; all from PeproTech) before chemotaxis, previously shown to be effective for desensitizing specific chemokine receptor responsiveness [123, 173]. For effector $CD8^+$ T cell chemotaxis, the lower chamber was filled with supernatants from 42 h co-cultures of NK cells and DCs, and the upper chamber was loaded with effector $CD8^+$ T cells ($2x10^5$) generated as described above. Cell numbers in the bottom chambers were assessed after 3 h by flow cytometry, and specific chemotaxis for each condition was calculated as the number of migrated cells subtracted by the number of migrated cells toward media-only controls.

Isolation of OvCa ascites cells. Human OvCa ascites were obtained intraoperatively from previously-untreated patients with advanced (stage III or IV) epithelial ovarian cancer undergoing primary surgical debulking for clinical staging. Written informed consent was

obtained prior to any specimen collection, and the nature and possible consequences of the studies were explained. All specimens were provided under a protocol approved by the University of Pittsburgh Institutional Review Board (IRB0406147). Primary OvCa ascites cells were harvested by centrifugation. NK cell-enriched and NK cell-depleted fractions were generated from bulk OvCa ascites cells by CD56 positive magnetic selection (StemCell Technologies).

Flow cytometry. Cell surface and intracellular immunostaining analyses were performed using an Accuri C6 Flow Cytometer. NK cells and T cells were stained with the dye-conjugated anti-human mouse monoclonal antibodies CD56-PE-Cy5 (Beckman Coulter), CD3-PE (eBioscience), Granzyme B-PE (Invitrogen), and CD16-FITC, CD8-PE-Cy5, CD45RA-FITC, CD45RO-PE, and CD57-FITC (BD Biosciences). Chemokine receptors on DCs and T cells were stained with the dye-conjugated anti-human mouse monoclonal antibodies CCR1-PE and CCR7-FITC (R&D Systems) and CCR5-FITC, CCR6-PE, CXCR1-FITC, CXCR3-PE, and CXCR4-PE (BD Biosciences), and the dye-conjugated anti-human goat polyclonal antibody XCR1-PE (R&D Systems). The corresponding mouse antibody isotype controls IgG1-FITC, IgG2a-FITC, IgG2b-FITC, IgG1-PE, IgG2a-PE, IgG2b-PE, and IgG1-PE-Cy5 (BD Biosciences) and normal goat antibody control IgG-PE (R&D Systems) were used, as appropriate. Before staining, the cells were treated for 20 min at 4ºC in PBS buffer containing 2% human serum, 0.5% BSA, 0.1% NaN_3, and 1 μg/ml of mouse IgG (Sigma-Aldrich) to block non-specific binding. Cell permeabilization for intracellular staining was performed using 0.1% Triton X-100 (Sigma) in PBS for 15 min. Cells were stained for 40 min at 4ºC followed by washing with PBS buffer containing 0.5% BSA and 0.1% NaN_3, then fixed and stored in 4% paraformaldehyde until analysis.

Quantitative real-time PCR. Analysis of mRNA expression was performed using the StepOne Plus System (Applied Biosystems), as previously described [173], using inventoried primer/probe sets. Preliminary kinetic analysis (data not shown) determined optimal expression of NK cell-expressed IFNγ, TNFα, CCL3, CCL4, CXCL8, and XCL1 at 4 h following cytokine stimulation of both purified NK cells and bulk OvCa ascites cells, and optimal expression of CXCL9 and CXCL10 in bulk OvCa ascites cells at 24 h following IL-18/IFNα stimulation. The expression of each gene was normalized to HPRT1 and expressed as fold increase ($2^{-\Delta CT}$), where $\Delta C_T = C_{T\,(\text{target gene})} - C_{T\,(\text{HPRT1})}$.

ELISA. Supernatants from 48 h co-cultures of NK cells and DCs were analyzed for CXCL9, CXCL10, CCL5, and CCL22 by indirect sandwich ELISA using specific matched primary and biotinylated-secondary antibody pairs (PeproTech), as previously described [173]. When indicated, DCs were harvested, washed, and re-plated in the presence of CD40L-transfected J558 cells (for rationale, see above), and 24 h culture supernatants were analyzed for levels of CXCL9, CXCL10, CCL5, and CCL22.

Statistical analysis. Data was analyzed using unpaired and paired t tests (two-tailed) and one-way and two-way ANOVA, where appropriate. Significance was judged at an α of 0.05.

3.4 RESULTS

3.4.1 Unique role of IL-18 in priming human NK cell attraction of DCs

While activated DCs have been previously reported to attract and activate NK cells [123, 327, 328], we observed that the ‘helper’ pathway of NK cell activation, induced by IL-18 and

secondary signals like tumor cells or IFNα [293, 297], strongly promoted NK cell attraction of autologous DCs, either directly isolated from peripheral blood or generated *in vitro* from monocyte precursors (Fig. 3.1A). In contrast, IL-2-induced 'killer' effector NK cells, characterized by enhanced cytotoxicity and ability to kill DCs [297], were not similarly effective in attracting DCs. Prompted by the essential role of the chemokine system in governing cellular interactions during immune responses [330, 331], we investigated the role of 'helper' NK cell-produced chemokines in their superior ability to attract DCs. Immature monocyte-derived DCs (iDCs) expressed similarly high levels of CCR5 and CXCR1 as blood-isolated DCs, as well as distinct but lower levels of CXCR4, with XCR1 expression limited to monocyte-derived iDCs (Fig. 3.1B and Appendix Fig. 4). Analysis of the expression of the known ligands for these DC chemokine receptors in NK cells revealed that the NK cells treated with either IL-2 or IL-18 alone expressed only limited levels of CCL3, CCL4, and CXCL8 (Fig. 3.1C), ligands for the chemokine receptors CCR1/5 and CXCR1, respectively, which are known to mediate migration of iDCs in peripheral tissues [29]. Similarly, NK cells treated with IL-2 or IL-18 alone showed minimal to modest enhancement, respectively, in the expression of XCL1, the ligand for the receptor XCR1 and a chemokine implicated in the attraction of DCs highly efficient in antigen cross-presentation [332, 333]. In contrast, combined stimulation of NK cells with IL-18 and IFNα, a factor recently shown to be important for initiating spontaneous anti-tumor immune responses *in vivo* [102, 103] and a secondary signal known to co-activate cytokine secretion in human 'helper' NK cells [203, 297], induced a strong synergistic enhancement in the expression of CCL3, CCL4, CXCL8, and XCL1 (Fig. 3.1C). Such two-signal induction of DC-attracting chemokines parallels the mode of induction of the DC-activating cytokines IFNγ and TNFα in human NK cells [203, 297]. Expression of the additional chemokines CCL20 and CXCL12,

ligands for the receptors CCR6 and CXCR4, were not observed in NK cells under any mode of stimulation (data not shown).

Additional NK cell subset analysis revealed that the $CD56^{dim}$ population was particularly responsive to IL-18-driven chemokine induction (Fig. 3.1D), while the $CD56^{bright}$ NK cell subset responded to IL-18 to a significantly lesser degree. This is consistent with prior work implicating the $CD56^{dim}$ subset as the predominant target of IL-18 in driving NK cell acquisition of lymph node-homing CCR7 and the early secretion of DC-polarizing IFNγ [297], as well as recent findings implicating the $CD56^{dim}$ subset as a major producer of cytokines and chemokines, particularly at early activation time-points [64, 65].

The ability to induce NK cell expression of DC-attracting chemokines was a specific feature of IL-18, since it could not be reproduced by IL-2 or IFNα (both known NK cell activating factors [300]) alone or in combination (Fig. 3.1C), nor by IL-1β, a member of the same family of cytokines as IL-18 (data not shown). The unique character of IL-18-induced 'helper' NK cells was further supported by the observation that only NK cells primed with IL-18 responded with enhanced expression of DC-attracting chemokines when exposed to such secondary stimuli as IFNα, IL-15, IL-12, and IL-2 (Fig. 3.2A). In contrast, providing these stimuli in reverse order (primary IL-2 followed by secondary stimulation with IL-18) was ineffective in inducing NK cell expression of DC-attracting chemokines, demonstrating that enhanced DC-recruiting function is a specific feature of IL-18-induced helper NK cells, rather than a general outcome of NK cell activation. IL-18 further primed NK cells for the expression of DC-attracting chemokines in response to multiple secondary pro-inflammatory cytokines (Fig. 3.2A) or in response to K562 tumor cells (Fig. 3.2B and Appendix Fig. 5), with the latter effect involving NKG2D-mediated recognition of tumor targets. These results indicate the role of IL-18

in priming NK cells for the attraction of DCs following subsequent NK cell exposure to such diverse signals as inflammatory cytokines and tumor cell recognition.

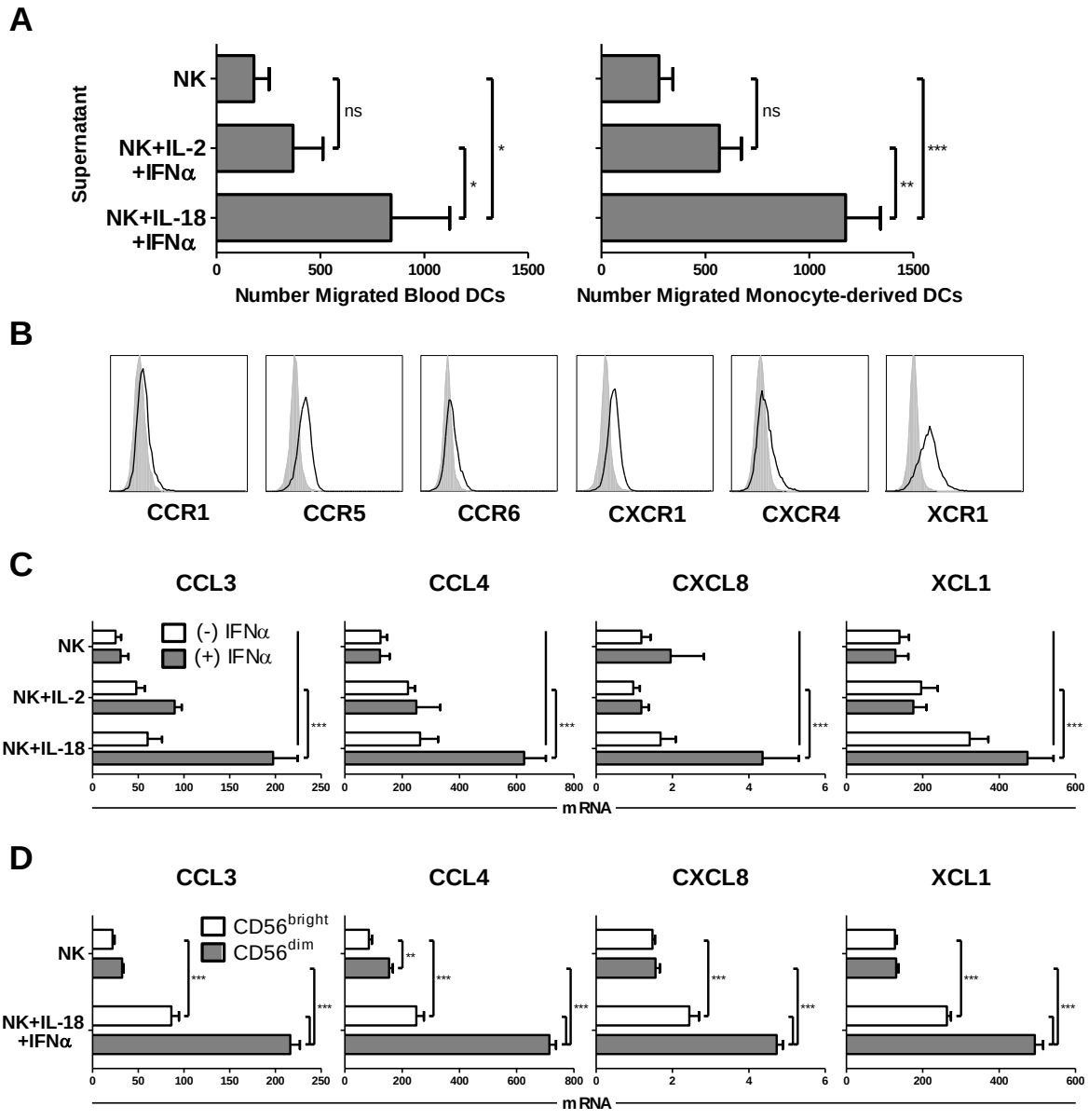

Figure 3.1. Unique role of IL-18 in priming human NK cell attraction of DCs.

(A) Chemotaxis of peripheral blood-isolated DCs (left) or day 6 monocyte-derived immature DCs (iDCs; right) toward culture supernatants from autologous NK cells stimulated for 36 h with IL-2 and IFNα or IL-18 and IFNα. Data shown represent the mean (± SD) number of specific migrated DCs from independent donors across independent experiments (3 donors for blood DCs; 5 donors for monocyte-derived DCs). (B) Surface expression

(open histograms) of CCR1, CCR5, CCR6, CXCR1, CXCR4, and XCR1 on monocyte-derived iDCs. Gray filled histograms represent isotype controls. (C) NK cells were incubated for 4 h in the presence of IL-2, IL-18, and/or IFNα, and subsequently analyzed for expression of the chemokines CCL3, CCL4, CXCL8, and XCL1. Data are expressed as ratios between the expression of individual chemokine genes and HPRT1, and represent the mean (± SD) of 5 independent donors. (D) Sorted CD56^{bright} and CD56^{dim} NK cells were incubated for 4 h in the absence or presence of IL-18/IFNα, and subsequently analyzed for expression of CCL3, CCL4, CXCL8, and XCL1. Data are expressed as ratios between the expression of individual chemokine genes and HPRT1, and shown as the mean expression (± SD) of triplicate cultures. Data represent one of two independent experiments, which both yielded similar results. ***p<0.001, **p<0.01, *p<0.05, ns: p>0.05 compared to indicated groups.

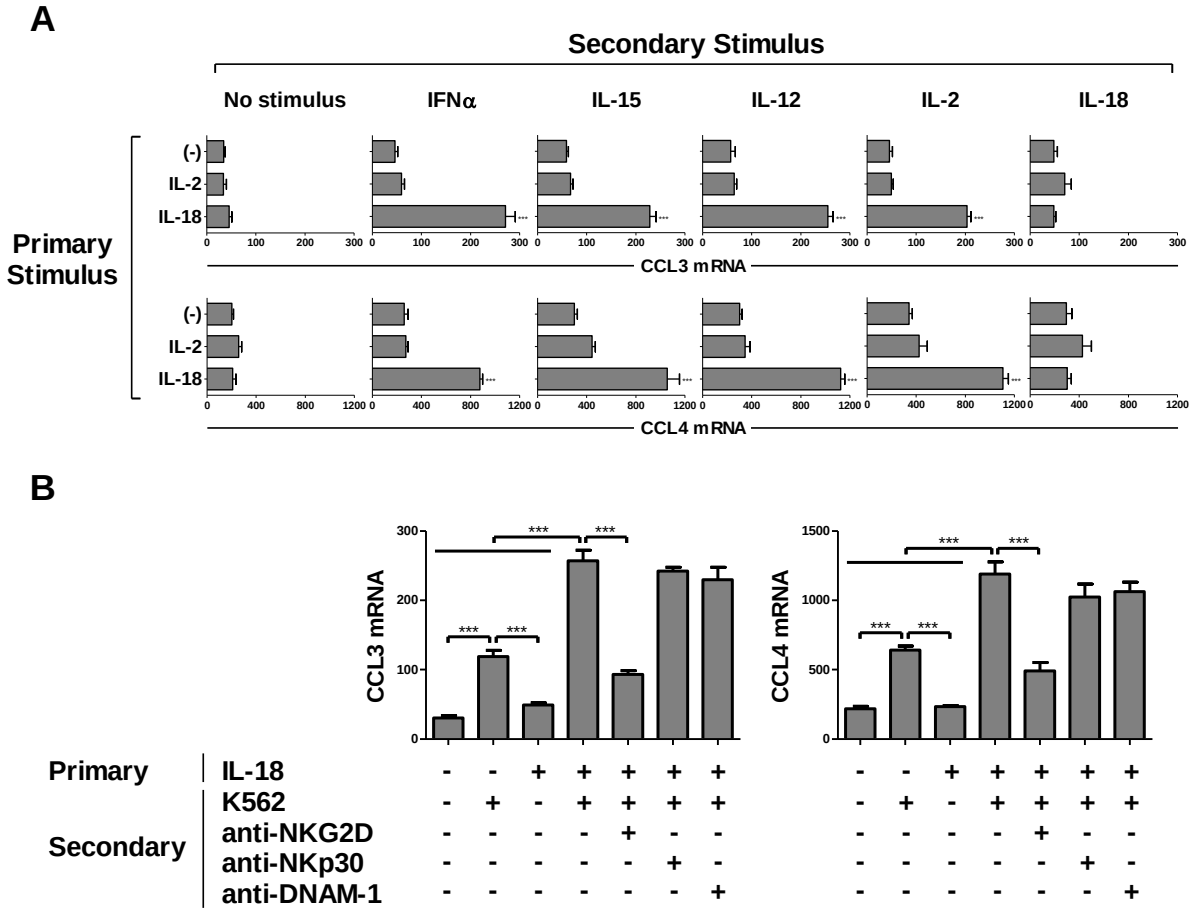

Figure 3.2. IL-18 synergizes with multiple secondary stimuli in inducing NK cell expression of DC-attracting chemokines.

(A) NK cells were pre-treated for 24 h in the absence or presence of IL-2 or IL-18, washed, and re-plated in the absence or presence of IFNα, IL-15, IL-12, IL-2, or IL-18. The expression of CCL3 (top) and CCL4 (bottom) were analyzed after 4 h incubation with the secondary stimulus. Data are expressed as ratios between the expression of individual chemokine genes and HPRT1, and recorded as the mean expression (± SD) assayed in triplicate cultures. Data represent one of three independent experiments, which all yielded similar results. (B) NK cells were pre-treated for 24 h in the absence or presence of IL-18, washed, and re-plated in the absence or presence of K562 cells (5:1 NK:K562 ratio). When indicated, NK cells were pre-treated for 30 min with blocking antibodies to NKG2D, NKp30, or DNAM-1 before co-culture with K562 cells. The expression of CCL3 (left) and CCL4 (right) were analyzed after 4 h activation with the secondary stimulus. Data are expressed as ratios between the expression of individual chemokine genes and HPRT1, and recorded as the mean expression (± SD) assayed in triplicate cultures. Data represent one of three independent experiments, which all yielded similar results. ***$p<0.001$ compared to indicated groups or compared to all groups when not specifically indicated.

3.4.2 Key role of CCR5 in the recruitment of autologous immature DCs by IL-18-primed NK cells

Desensitization of specific chemokine receptors on iDCs with a large panel of DC and NK cell-relevant chemokines (Fig. 3.3A; see Materials and Methods for discussion of the technique) revealed a highly selective role for CCR5, but not CCR6, XCR1, CXCR1, or CXCR4, in the recruitment of autologous iDCs by IL-18-primed NK cells. Specific antibody blockade of the CCR5 receptor (Fig. 3.3B) confirmed the key role of this receptor in helper NK cell-mediated iDC attraction.

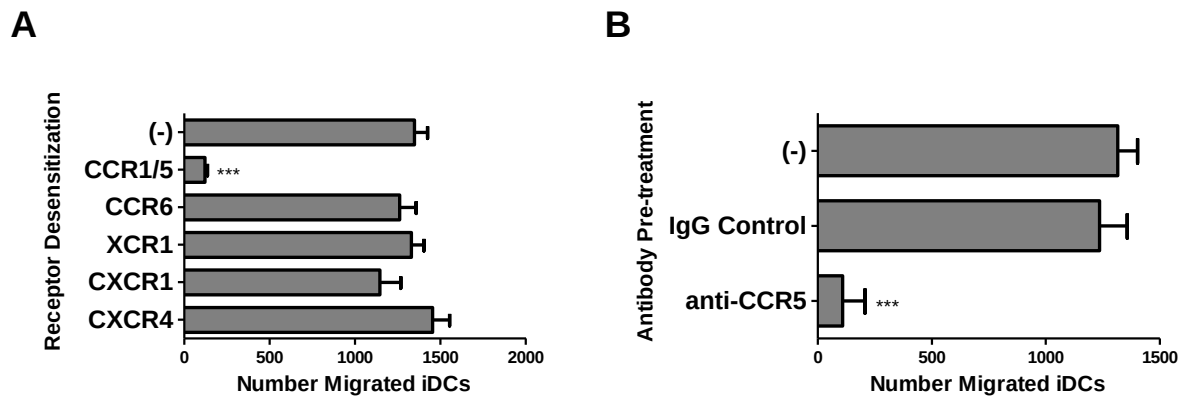

Figure 3.3. IL-18-primed NK cells attract autologous immature DCs through a CCR5-dependent mechanism. (A-B) iDCs were pre-treated for 30 min with the chemokines CCL3, CCL20, XCL1, CXCL8, or CXCL12 (A) to block the DC chemokine receptors CCR1/5, CCR6, XCR1, CXCR1, or CXCR4, respectively, or treated with a blocking anti-CCR5 monoclonal antibody (B) before migration toward 36 h supernatants collected from IL-18/IFNα-stimulated autologous NK cells. Data is shown as mean (± SD) number of specific migrated iDCs in triplicate cultures. Data shown was obtained from one representative experiment of three performed, all yielding similar results. ***$p<0.001$ compared to all groups.

3.4.3 IL-18-primed NK cells collaborate with DCs in the recruitment of effector $CD8^+$ T cells

Co-culture of IL-18-primed NK cells with autologous iDCs resulted in highly elevated levels of CXCL9, CXCL10, and CCL5 (Fig. 3.4A, top), the chemokines that have been implicated in the attraction of type-1 T_{eff} cell subsets central to efficient anti-tumor responses [44-46]. This effect was not accompanied by an increase in the secretion of CCL22, a regulatory T cell-attracting chemokine [47]. The induction of these T_{eff} cell-recruiting chemokines in NK-DC co-cultures was dependent on TNFα and IFNγ, since the enhanced chemokine secretion was abrogated upon addition of soluble TNF and IFNγ decoy receptors to the co-cultures (Fig. 3.4A, top). Elevated

production of T_{eff} cell-recruiting chemokines by NK cell-activated DCs was maintained even after subsequent harvesting, washing, removal of NK cells, and re-stimulation of the DCs with CD40L (Fig. 3.4A, bottom), demonstrating the long-term impact of IL-18-primed NK cells on DC chemokine production.

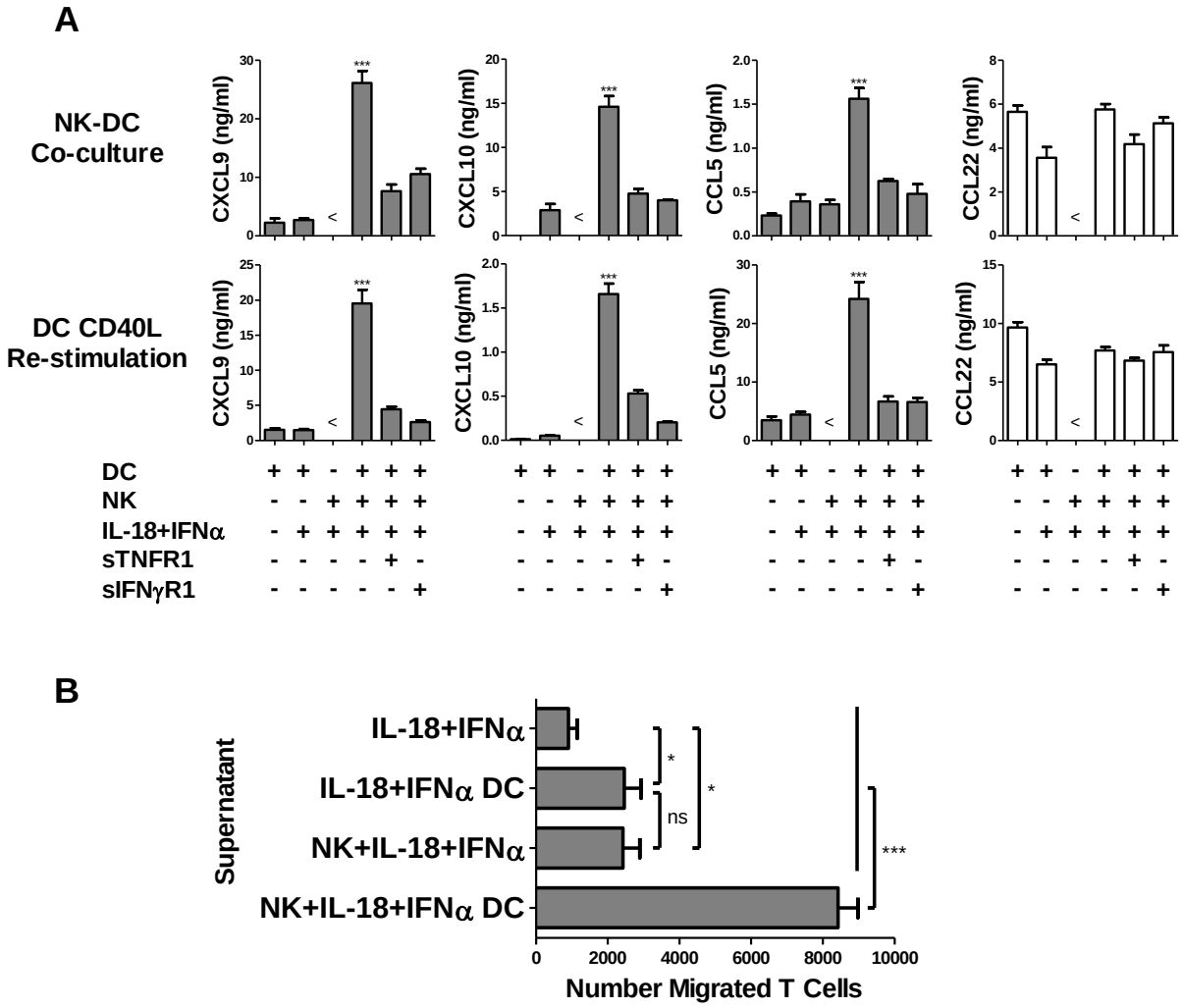

Figure 3.4. IL-18-primed NK cells induce DC production of T_{eff} cell-recruiting chemokines, promoting T_{eff} cell attraction.

NK cells were added to autologous day 6 DCs (1:2 NK:DC ratio) in the presence of IL-18 and IFNα. After 48 h, co-culture supernatants were harvested for analysis and chemotaxis experiments, and DCs were harvested, washed, depleted of NK cells, and re-stimulated with CD40L for 24 h. (A) CXCL9, CXCL10, CCL5, and CCL22 levels in

supernatants of untreated immature DCs (iDCs) or DCs exposed to IL-18/IFNα with or without autologous NK cells, in the additional presence or absence of soluble TNF (sTNFR1) or IFNγ (sIFNγR1) decoy receptors, after 48 h co-culture (top) or following harvesting, washing, NK cell depletion, and 24 h CD40L stimulation (bottom). (B) Migration of effector $CD8^+$ T cells (see Materials and Methods for generation) toward supernatants collected from 48 h cultures of IL-18/IFNα alone, NK cells treated with IL-18/IFNα, or DCs exposed to IL-18/IFNα with or without autologous NK cells. Data recorded as mean (± SD) in triplicate cultures from one representative experiment of three performed, all yielding similar results. ***$p<0.001$, *$p<0.05$, ns: $p>0.05$ compared to indicated groups or compared to all groups when not specifically indicated. < indicates levels were below the limit of detection of the assay.

Supernatants from NK cell-activated DCs were highly efficient at recruiting T_{eff} cells (Fig. 3.4B). Importantly, although supernatants from two-signal-activated NK cells alone or IL-18/IFNα-activated DCs alone were capable of mild T_{eff} cell attraction over baseline, the supernatants generated from NK-DC interaction had a greatly- and synergistically-enhanced capacity for T_{eff} cell recruitment, demonstrating the key role of DCs in NK cell-initiated T_{eff} cell attraction.

3.4.4 NK cell-mediated enhancement of T_{eff} cell-recruiting chemokines can be induced in the human ovarian cancer environment

In order to assess the potential for utilizing NK 'helper' cell paradigms in the therapy of cancer patients, we evaluated the ability of IL-18 and NK cells to regulate chemokine production in the malignant ascites of patients with stage III-IV epithelial ovarian cancer. Similar to what has been previously reported [334], significant populations of $CD3^-CD56^+$ NK cells were found within the ascites cells (NK cells as percentage of tumor-associated lymphocytes: median=15.42%;

range=2.1-30.5%; n=4). Although tumor-associated NK cells can demonstrate reduced functionality [22, 135], we observed that NK cells freshly isolated from malignant ovarian ascites could be effectively activated by IL-18/IFNα to express the NK helper-associated DC-attracting and DC-activating factors CCL3, CCL4, IFNγ, and TNFα (Fig. 3.5A).

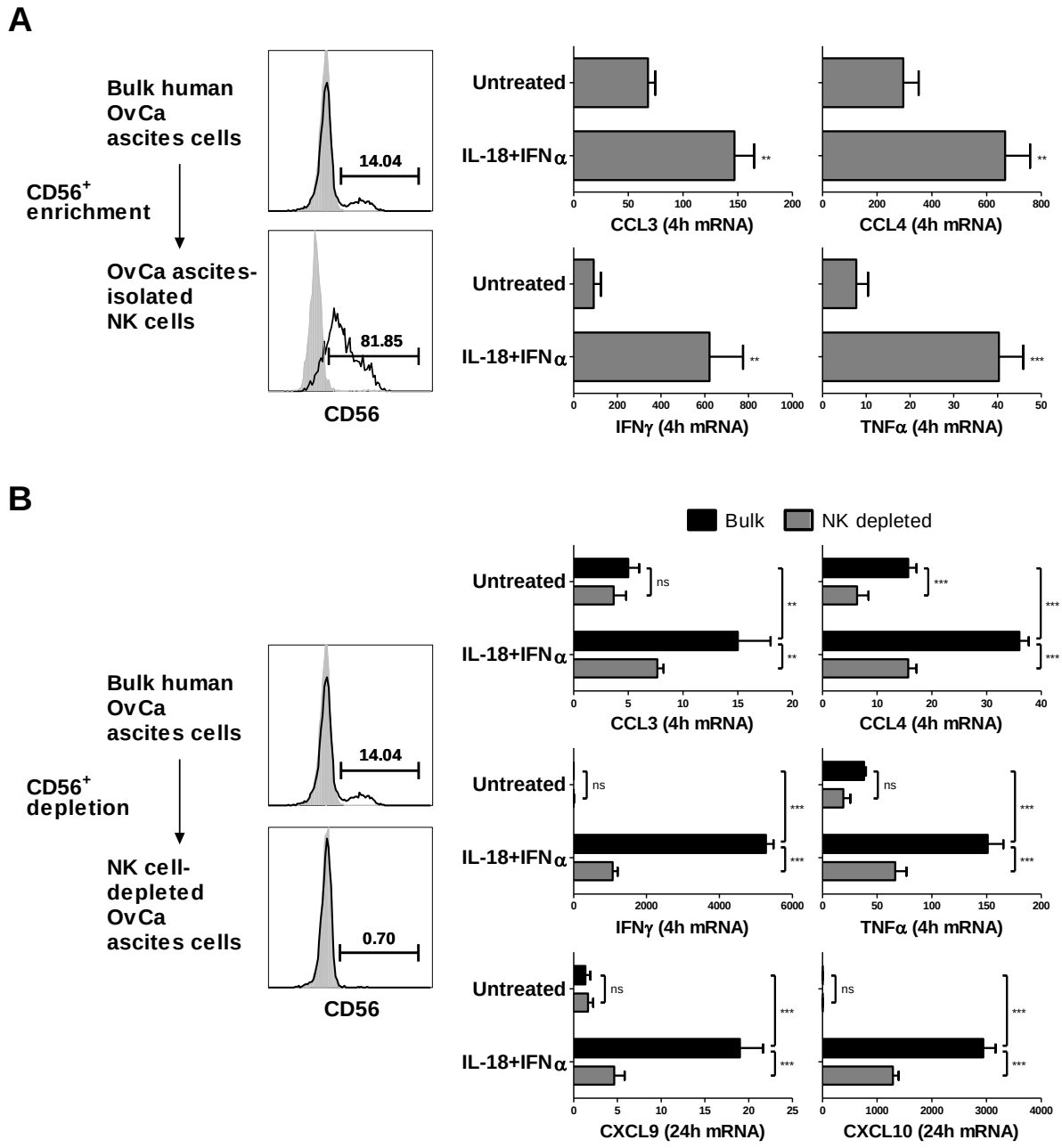

Figure 3.5. NK cells drive T_{eff} cell-recruiting chemokines in the human ovarian cancer environment.

(A) $CD56^+$ NK cells were enriched from human ovarian cancer (OvCa) ascites cells (left), cultured for 4 h in the absence or presence of IL-18/IFNα, and analyzed for expression of the chemokines CCL3 and CCL4 and the cytokines IFNγ and TNFα (right). (B) Bulk OvCa ascites cells or ascites cells depleted of $CD56^+$ NK cells (left) were cultured for 4 or 24 h in the absence or presence of IL-18/IFNα, and analyzed for expression of the chemokines CCL3, CCL4, CXCL9, and CXCL10 and the cytokines IFNγ and TNFα (right). Flow cytometric analyses of NK cell enrichment and depletion are representative of one of four patients. Gene expression data are expressed as ratios between the expression of individual chemokine or cytokine genes and HPRT1, and represent the mean (± SD) across four independent experiments using ascites cells from four different patients. ***$p<0.001$, **$p<0.01$, ns: $p>0.05$.

Furthermore, IL-18/IFNα treatment of bulk ascites cells induced the production of high levels of the DC activators IFNγ and TNFα and the T_{eff} cell-recruiting chemokines CXCL9 and CXCL10 (Fig. 3.5B), demonstrating the ability of IL-18/IFNα–activated NK cells to function even in the suppressive environment of OvCa. Depletion of NK cells from the bulk ascites cell population suppressed the production of these chemokines and cytokines mediated by IL-18/IFNα treatment, indicating that the activation of tumor-associated NK cells by the NK 'helper'-driving stimuli plays the key role in the induction of DC-attracting, DC-activating, and T_{eff} cell-recruiting factors within the human cancer environment.

3.5 DISCUSSION

While IL-18-induced NK cells can promote antitumor immunity by elevating the production of Th1- and CTL-driving IL-12 by local DCs, our current data indicate their additional role in promoting DC attraction and the conditioning of tumor sites for the chemokine-driven infiltration

of desirable effector-type T cells. We demonstrate that chemokine production is not a general consequence of NK cell activation, but is strictly regulated and selectively associated with a 'helper' NK cell phenotype driven by IL-18. Although IL-18 and IL-2 are both known to be potent NK cell activating factors, these data indicate that only IL-18 is efficient in enhancing NK cell expression of the DC-attracting chemokines CCL3, CCL4, CXCL8, and XCL1, resulting in the attraction of immature DCs. Such IL-18-driven enhancement in the expression of DC-attracting chemokines corresponds closely to the previously-reported regulation of IFNγ and TNFα, factors essential for NK cell-mediated activation of DCs, in IL-18-primed human NK cells [297]. This suggests that NK cell recruitment of DCs and NK cell-mediated activation of DCs are closely related phenomena governed by similar mechanisms, supporting the role of NK cells as important modulators of DC-mediated immune responses.

Interestingly, the unique 'priming' effects of IL-18 in promoting 'helper' NK cell-driven chemokine interactions with DCs requires secondary stimulation with other pro-inflammatory factors (see Fig. 3.2A) or recognition of target cells (see Fig. 3.2B and Appendix Fig. 5). These secondary signals, described in this study and others [297, 335], can include direct interaction with tumor cells or type I interferons likely to be elaborated early in viral infection. This two-signal requirement for IL-18-primed NK cell function is likely to represent a critical checkpoint in NK cell activation, preventing inappropriate development of potent downstream immune responses, including those initiated through NK cell interaction with DCs. Although IL-18 has been reported to favor protective anti-tumor immunity [336, 337], IL-18 has also been recently implicated in tumor-associated immunosuppression through its promotion of a Kit^+ subset of regulatory NK cells overexpressing PD-L1 [338, 339]. Therefore, it is possible that in some situations, IL-18-primed NK cells may mediate different functions depending on the availability

and/or character of associated secondary signals. Indeed, the activity of IL-18 has been shown to be highly context-dependent, and demonstrates the capacity to co-induce either the type-1 cytokine IFNγ or the type-2 cytokine IL-13 when combined with different secondary signals [340]. The differential impact of distinct secondary signals specifically on IL-18-primed NK cell activity is the subject of our current ongoing investigations.

The quantity and quality of immune cell infiltration into the tumor environment, including the critical balance between effector and regulatory T cells, have been increasingly recognized as vital components of both spontaneous and therapy-induced anti-tumor immune control [9, 341]. Importantly, the chemokines CXCL9, CXCL10, and CCL5 have been implicated in the attraction of type-1 T_{eff} cell subsets central to effective anti-tumor responses [44-46], providing key targets for therapeutic 'conditioning' of the tumor chemokine environment for efficient anti-tumor effector cell entry. Although this study demonstrates that IL-18-primed NK cells can directly express CCR5 ligands, their role in generating a chemokine environment conducive to type-1 T_{eff} cell recruitment is most likely to occur through their activity on DCs, given the apparent strong synergy between IL-18-driven NK cells and DCs in promoting T_{eff} cell attraction that was significantly more efficient than NK cells or DCs alone (see Fig. 3.4B).

Notably, this study demonstrates that IL-18-primed human NK cells, including from directly within the human cancer environment, can enhance type-1 immune responses by selectively inducing high DC expression of T_{eff} cell-recruiting chemokines, including CXCL9, CXCL10, and CCL5, without inducing the T_{reg} cell-attracting chemokine CCL22. However, intratumoral NK cells have also been shown to be capable of secreting CCL22 and mediating the recruitment of $CD4^+CD25^+FoxP3^+$ regulatory T cells, a process which can be driven by NK cell

activation with IL-2 [342]. This highlights the importance of carefully defining the relevant NK cell stimulatory factors for the therapeutic augmentation of intratumoral immune control.

In addition to their expression by T_{eff} cells, resting NK cells have also been shown to express the chemokine receptors CCR5 and CXCR3, and can respond to their respective ligands produced by the interaction between IL-18-primed NK cells and DCs [343]. Since DCs can be an important source of IL-18 during developing immune responses [325], and mature DCs can play a significant role in activating resting NK cells [98], this presents the possibility of a reciprocal, chemokine-driven feed-forward interaction between NK cells and DCs, in which NK cell-activated DCs can subsequently attract and activate additional resting NK cells, further promoting an amplifying cycle of immune activation. Indeed, reciprocal positive feedback has been demonstrated between NK cells and myeloid cells, including DCs [98], and spatial innate cell clustering has been shown to be important to developing protective immune responses *in vivo* [184]. Our current data suggests that the suppressive nature of the human tumor environment may not, at least, represent an absolute, irreversible barrier to NK cell activation toward DC-stimulating helper function, and may be amendable to therapeutic modulation, for instance through the local application of IL-18 and IFNα. Thus, the 'helper' interaction between IL-18-primed NK cells and DCs may represent a powerful feed-forward loop amplifying endogenous immune responses, and may present an attractive target for cancer therapy in which modest initiation of the helper response may result in a much larger induction of effector activity.

In summary, these data identify the unique chemokine expression of 'helper' versus 'killer' pathways of NK cell differentiation, and demonstrates that human NK cells can serve important helper functions in facilitating the chemokine-driven attraction and activation of DCs and the accumulation of effector cells in the tumor environment. This study further demonstrates

that NK cells in cancer patients, including NK cells infiltrating the tumor environment itself, are competent to undergo helper differentiation, and thus may serve as therapeutic targets for the modulation of the human tumor chemokine environment to facilitate type-1 immune responses against cancer.

4.0 HELPER NK CELLS PROMOTE T CELL PRIMING BY INDUCING DC PRODUCTION OF CCL19

Adapted from:

Human 'helper' NK cells promote T cell priming by inducing dendritic cell production of CCL19

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These data are reported as a Manuscript in Preparation.

4.1 ABSTRACT

Effective accumulation and interaction of mature dendritic cells (DCs) and naïve T cells within lymph nodes driven by the CCR7-CCL19/CCL21 axis are critical for the induction of adaptive T cell immunity. Human natural killer (NK) cells activated by IL-18 exhibit unique 'helper' activity in promoting productive DC-T cell interactions, inducing dendritic cell (DC) maturation and the type-1-polarization of DC-primed T cell responses. Here we demonstrate that such IL-18-induced 'helper' NK cells, upon secondary stimulation with the additional inflammatory signals IFNα, IL-15, IL-12, or IL-2, uniquely induce high DC production of CCL19 in a TNFα and IFNγ-dependent mechanism. Helper NK cell-activated DCs promote efficient CCR7-mediated recruitment of naïve $CD8^+$ T cells, inducing their expansion and acquisition of granzyme B. Using lymph nodes isolated from colorectal cancer patients, we further demonstrate enhanced expression of CCL19 in human tumor-associated lymphoid tissue induced by treatment with helper NK cell-stimulating factors. Our data demonstrate the ability of two-signal-activated 'helper' NK cells to promote lymph node production of the DC- and naïve/memory T cell-attracting chemokine CCL19, providing rationale for NK cell-targeting using IL-18-containing combinatorial adjuvants to promote the induction of adaptive anti-tumor immunity.

4.2 INTRODUCTION

Effective induction of T cell responses requires the productive lymph node association of activated DCs with naïve and memory T cells. Interaction of the chemokine receptor CCR7 and its ligands CCL19 and CCL21 play essential roles in this process, governing the entry of both

CCR7-expressing naïve T cells and activated DCs into the lymph node (LN) [31, 32], their co-localization within the T cell zones of the LN paracortex [33], and their effective dynamic interaction [34-37]. The CCR7-CCL19/CCL21 axis has likewise been implicated in the optimal recruitment of central memory T cells to the LN [344, 345], positioning them for efficient DC activation of recall responses.

NK cells have been demonstrated to play key 'helper' roles in directing DC-mediated priming of adaptive T cell immunity [123, 202, 204]. In particular, NK cells activated by IL-18, a cytokine elaborated early in response to tissue damage, infection, or transformation [324], have been demonstrated to uniquely localize to sites of DC-T cell interaction and provide an important early source of IFNγ and TNFα, promoting DC maturation and polarizing DC-mediated T cell priming toward type-1 helper $CD4^+$ T cell (Th1) and cytotoxic $CD8^+$ T cell (CTL) responses [124, 297, 346]. While 'helper' NK cells have been shown to direct DCs to draining LNs and facilitate the priming of naïve T cells [326], it remains unknown whether NK cells also promote T cell recruitment to the lymph nodes and their effective interaction with DCs.

Here we show that human two-signal-activated 'helper' NK cells, induced uniquely by exposure to IL-18 and secondary pro-inflammatory signals, instruct DCs to secrete high levels of the CCR7 ligand CCL19, driving efficient DC recruitment of naïve T cells and subsequent T cell expansion and acquisition of effector molecules. Importantly for the prospective clinical application of NK cell-targeting 'binary adjuvants' involving IL-18, we further demonstrate that treatment of lymph nodes from colorectal cancer patients with such NK cell-activating stimuli is capable of inducing high expression of CCL19 within human lymphoid tissues.

4.3 MATERIALS AND METHODS

Media and reagents. Serum-free CellGenix DC medium (CellGenix Technologie Transfer GmbH) was used for short-term culture of human NK cells and for DC generation. Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum and 1% L-glutamine and penicillin/streptomycin (all from Gibco, Invitrogen) was used as the base medium for the outgrowth of T cell cultures, the culture of human lymph node explants, and for the maintenance of the J558-CD40L tumor cell line. The following factors were used throughout the study: IL-18 (MBL International); IL-2 (Chiron); IFNα (Intron A, IFN-α-2b; Schering-Plough); IL-12 (PeproTech); IL-15 (Sigma-Aldrich); and granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (Schering-Plough).

NK cell and $CD8^+$ T cell isolation. Peripheral blood from healthy donors was harvested by venipuncture under IRB-approved protocols, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation using Lymphocyte Separation Medium (Cellgro Mediatech). NK cells ($CD56^+CD3^-$) and naïve $CD8^+$ T cells ($CD8^+CD45RA^+CCR7^{high}CD45RO^-CD56^-CD57^-$) were further isolated from PBMCs by negative magnetic selection (>95% pure in both cases) using the EasySep system (StemCell Technologies), according to the manufacturer's protocol.

Generation of DCs. Monocyte fractions were further isolated from PBMCs by CD14 positive magnetic selection (Miltenyi Biotech). Immature DCs were generated from monocytes cultured for 6 days in 24-well plates at $4x10^5$ cells/well in GM-CSF and IL-4 (both 1000 IU/ml).

NK cell activation of DCs. Previously isolated and cryopreserved autologous NK cells were thawed and added to DC cultures at $1.5x10^5$ cells/well to day 6 DC cultures (1:2 NK:DC ratio) in the presence of IL-18 (200 ng/ml) or IL-2 (250 IU/ml) together with IFNα (1000 IU/ml),

IL-12 (5 ng/ml), IL-2 (250 IU/ml), IL-15 (100 ng/ml), or IL-18 (200 ng/ml). Alternatively, NK cells were pre-treated with IL-18 or IL-2 for 24 h, washed thoroughly, and re-plated with DCs in the presence of IFNα, IL-12, IL-2, IL-15, or IL-18 as a secondary stimulus. When indicated, soluble decoy receptors to TNFα (sTNFR1; 1 μg/ml; R&D Systems) and IFNγ (sIFNγR1; 10 μg/ml; R&D Systems) were added to cultures at co-culture initiation. Supernatants were collected at 48 h for CCL19 analysis by specific ELISA (PeproTech). To assess the stability of DC chemokine production, NK-DC co-cultures were harvested and washed, NK cells were removed by CD56 positive magnetic selection (StemCell Technologies), and DCs were then re-plated in 96-well plates at $2x10^4$ cells/well. To mimic interaction with CD40L-expressing $CD4^+$ T cells, DCs were co-cultured with CD40L-transfected J558 cells (a gift from Dr. P. Lane, University of Birmingham, United Kingdom) at $5x10^4$ cells/well, which in previous studies proved equivalent to activated $CD4^+$ T cells and soluble CD40L [175]. Supernatants were collected after 24 h and analyzed by specific ELISA for CCL19 (PeproTech).

ELISA. Supernatants from 48 h co-cultures of NK cells and DCs were analyzed for CCL19 by indirect sandwich ELISA using specific matched primary and biotinylated-secondary antibody pairs (PeproTech), as previously described [304]. When indicated, DCs were harvested, washed, and re-plated in the presence of CD40L-transfected J558 cells (for rationale, see above), and 24 h culture supernatants were analyzed for levels of CCL19. Supernatants from human lymph node explants (see below) treated for 24 h with IL-18/IFNα were similarly analyzed for CCL19.

Chemotaxis. Chemotaxis assays were performed using 24-(Trans)well plates with 5 μm pore-size polycarbonate membranes (Corning), as previously described [173]. The lower chamber was filled with supernatants from 48 h co-cultures of NK cells and DCs, and the upper

chamber was loaded with naive $CD8^+$ T cells ($2x10^5$) isolated as described above. When indicated, T cells were treated for 30 min with an anti-CCR7 blocking antibody (Clone 3D12, 20 μg/ml; BD Biosciences) before chemotaxis to block CCR7-dependent migration. Migrated cells in the bottom chambers were harvested after 3 h and re-suspended in 100 μl of 4% paraformaldehyde, and cell numbers per 60 μl volume were assessed by flow cytometry. Specific chemotaxis for each condition was calculated as the number of migrated cells subtracted by the number of migrated cells toward media-only controls.

***In vitro* priming of migrated naïve $CD8^+$ T cells.** Naïve $CD8^+$ T cells labeled with CFSE (Invitrogen; labeled according to the manufacturer's protocol) were allowed to migrate toward DC supernatants, as described above. Migrated T cells were pooled from triplicate chemotaxis wells and then re-plated with their respective DCs ($2x10^4$ DC per well in 96-well plates), which had been pulsed with staphylococcal enterotoxin B (SEB; 1 ng/ml) for 30 min. IL-2 (20 IU/ml) was added to cultures on day 5, and T cells were analyzed by flow cytometry on day 7 for cell numbers, proliferation by CFSE dilution, and acquisition of intracellular granzyme B.

Flow cytometry. Cell surface and intracellular immunostaining analyses were performed using an Accuri C6 Flow Cytometer. NK cells and T cells were stained with the dye-conjugated anti-human mouse monoclonal antibodies CD56-PE-Cy5 (Beckman Coulter), CD3-PE (eBioscience), CCR7-FITC (R&D Systems), granzyme B-PE (Invitrogen), and CD16-FITC, CD8-PE-Cy5, CD45RA-FITC, CD45RO-PE, and CD57-FITC (BD Biosciences). The corresponding mouse antibody isotype controls IgG1-FITC, IgG2b-FITC, IgG1-PE, IgG2a-PE, and IgG1-PE-Cy5 (BD Biosciences) were used, as appropriate. Before staining, the cells were treated for 20 min at 4ºC in PBS buffer containing 2% human serum, 0.5% BSA, 0.1% NaN_3,

and 1 μg/ml of mouse IgG (Sigma-Aldrich) to block non-specific binding. Cell permeabilization for intracellular staining was performed using 0.1% Triton X-100 (Sigma) in PBS for 15 min. Cells were stained for 40 min at 4ºC followed by washing with PBS buffer containing 0.5% BSA and 0.1% NaN_3, then fixed and stored in 4% paraformaldehyde until analysis.

***Ex vivo* culture of human lymph node explants.** Lymph nodes were obtained from colorectal cancer patients undergoing standard-of-care surgical treatment. Written informed consent was obtained prior to any specimen collection, and the nature and possible consequences of the studies were explained. All specimens were provided under a protocol approved by the University of Pittsburgh Institutional Review Board (UPCI 02-077). Lymph node tissue was sectioned using a 4 mm biopsy puncher and placed in IMDM containing 10% fetal bovine serum and 1% L-glutamine and penicillin/streptomycin. When indicated, the tissues were treated with IL-18 (200 ng/ml) and IFNα (1000 IU/ml). After 24 h incubation, supernatant was collected for ELISA analysis and tissue was analyzed for mRNA expression (see below).

Quantitative real-time PCR. Lymph node tissue was placed in Lysing Matrix E Tubes (MP Biologicals) containing RLT lysis buffer (Qiagen) and agitated using a FP120 homogenizer (MP Biologicals). Supernatant was collected and total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's protocol. Analysis of mRNA expression was performed using the StepOne Plus System (Applied Biosystems), as previously described [173], using inventoried primer/probe sets. Expression of TNFα, IFNγ, and CCL19 was assessed at 24 h following IL-18/IFNα stimulation. The expression of each gene was normalized to HPRT1 and expressed as fold increase ($2^{-\Delta CT}$), where $\Delta C_T = C_{T\,(\text{target gene})} - C_{T\,(\text{HPRT1})}$.

Statistical analysis. Data was analyzed using unpaired and paired t tests (two-tailed) and one-way and two-way ANOVA, where appropriate. Significance was judged at an α of 0.05.

4.4 RESULTS

4.4.1 Two-signal-activated ‘helper’ NK cells induce high CCL19 production by autologous DCs

We have previously reported the independent regulation of ‘effector’ versus ‘helper’ pathways of human NK cell differentiation, in which IL-2 preferentially promotes an ‘effector’ phenotype characterized by enhanced cytotoxicity, including against DCs, while IL-18 instead induces a distinct ‘helper’ differentiation pathway selectively supporting DC-mediated T cell priming via enhanced DC maturation, co-stimulatory molecule expression, and IL-12 production for the development of Th1 and CTL responses [297]. Given the critical role of chemokines, particularly CCL19 and CCL21, in directing interactions between DCs and naïve T cells in lymphoid tissue [347], we hypothesized that differential IL-2- versus IL-18-driven human NK cell differentiation may also differentially regulate NK cell ability to modulate DC expression of chemokines involved in the recruitment of naïve T cells, facilitating their unique outcomes on DC-mediated T cell priming.

In direct NK-DC co-culture, we observed that NK cell activation with IL-2 or IL-18 alone had no effect on levels of CCL19 in co-culture supernatants (Fig. 4.1A, left). In contrast, combined stimulation with IL-18 and IFNα, another early factor produced in response to viral infection and developing tumors [101, 348] and a known co-activator of human NK cell cytokine secretion [203], induced the synergistic enhancement of CCL19 production. This two-signal induction of CCL19 was maintained even after harvesting, washing, removal of NK cells, and re-stimulation of the DCs with CD40L (Fig. 4.1A, right), indicating the stable priming of DCs for high CCL19 production even after the initial NK-DC interaction, including upon subsequent

interaction with CD40L-expressing $CD4^+$ T cells. No CCL19 production was detected in cultures of IL-18/IFNα-activated NK cells alone and only limited levels in cultures of IFNα- or IL-18/IFNα-activated DCs alone (Fig. 4.1A and Fig. 4.2), confirming DCs as the source of CCL19 as well as the strict requirement for NK-DC interaction in its induction. Although other chemokines, including CCL21 and CXCL12, have been reported to interact with naïve T cells [349], no expression of these chemokines by DCs could be detected under any conditions (data not shown), in agreement with previous reports [350-352].

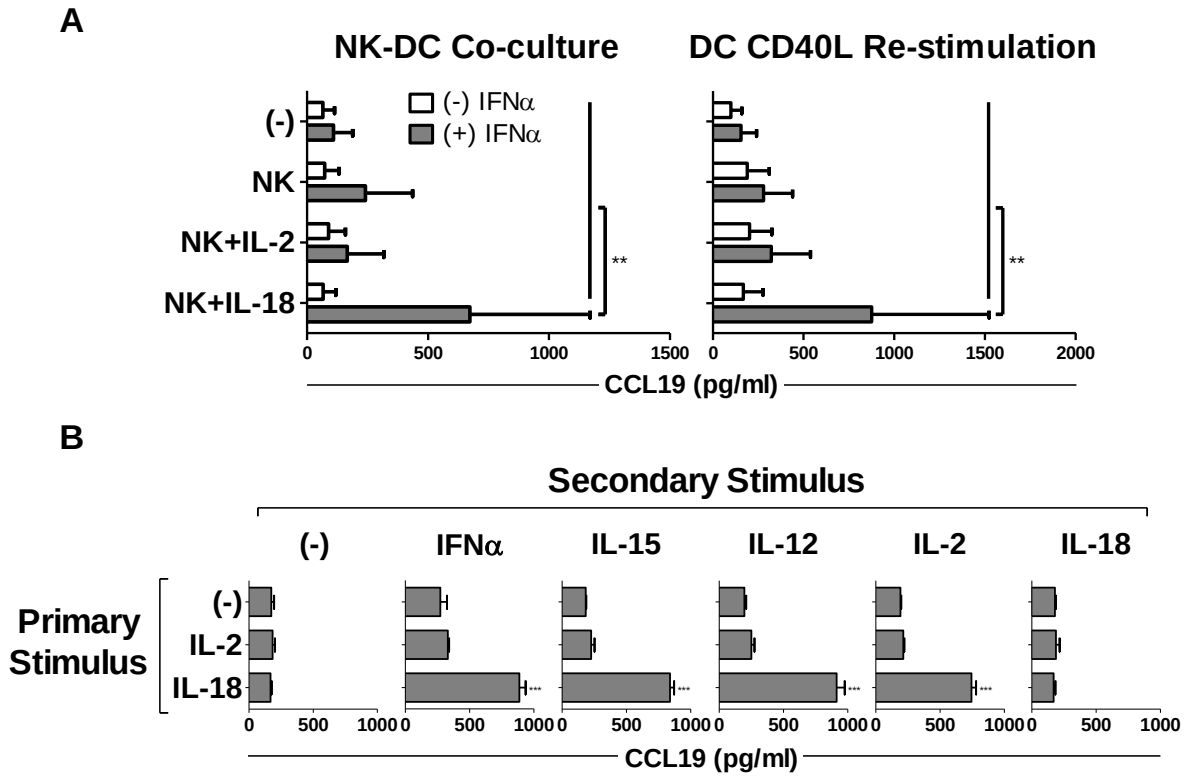

Figure 4.1. IL-18-primed NK cells induce DC production of CCL19.

(A) CCL19 levels in 48 h culture supernatants (left) of untreated DCs or DCs exposed to autologous NK cells (1:2 NK:DC ratio) in the presence of IL-2, IL-18, and/or IFNα; or in 24 h culture supernatants (right) of NK cell-activated DCs alone after harvesting, washing, depletion of NK cells, and re-stimulation with CD40L. Data recorded as the mean (± SD) of 6 independent donors. (B) NK cells were pre-treated for 24 h in the absence or presence of IL-2 or IL-18, washed, and re-plated with autologous DCs in the absence or presence of IFNα, IL-15, IL-12, IL-2, or

IL-18. Levels of CCL19 were analyzed after 48 h incubation with the secondary stimulus. Data are recorded as the mean (± SD) of triplicate cultures. Data represent one of three independent experiments, which all yielded similar results. ***$p<0.001$, **$p<0.01$ compared to indicated groups or compared to all groups when not specifically indicated.

Importantly, this CCL19 induction was shown to critically depend on NK cell activation with IL-18, as a similar enhancement was not observed with the known NK cell activating factors IL-2 or IFNα [300] either alone or in combination (Fig. 4.1A). Furthermore, only NK cells activated with IL-18 (but not IL-2) induced DC production of CCL19 upon exposure to a diverse range of secondary stimuli such as IFNα, IL-15, IL-12, or IL-2 (Fig. 4.1B). Interestingly, while the effective induction of CCL19 during NK-DC interaction could be induced by priming NK cells with IL-18, followed by secondary exposure to additional stimuli including IL-2, IL-2-primed NK cells could not induce DC production of CCL19 even upon secondary stimulation with IL-18 (Fig. 4.1B).

4.4.2 Key role of NK cell-derived TNFα and IFNγ in inducing autologous DC production of CCL19

Since the ability of two-signal activated NK cells to induce DC maturation has been shown to involve NK cell-produced TNFα and IFNγ [203, 297], we tested whether these factors may also mediate the NK cell induction of DC-produced CCL19. Indeed, NK-DC co-cultures in the presence of soluble decoy receptors to TNFα and IFNγ resulted in significant decreases in CCL19 levels, indicating the key role of these factors in the CCL19 induction (Fig. 4.2).

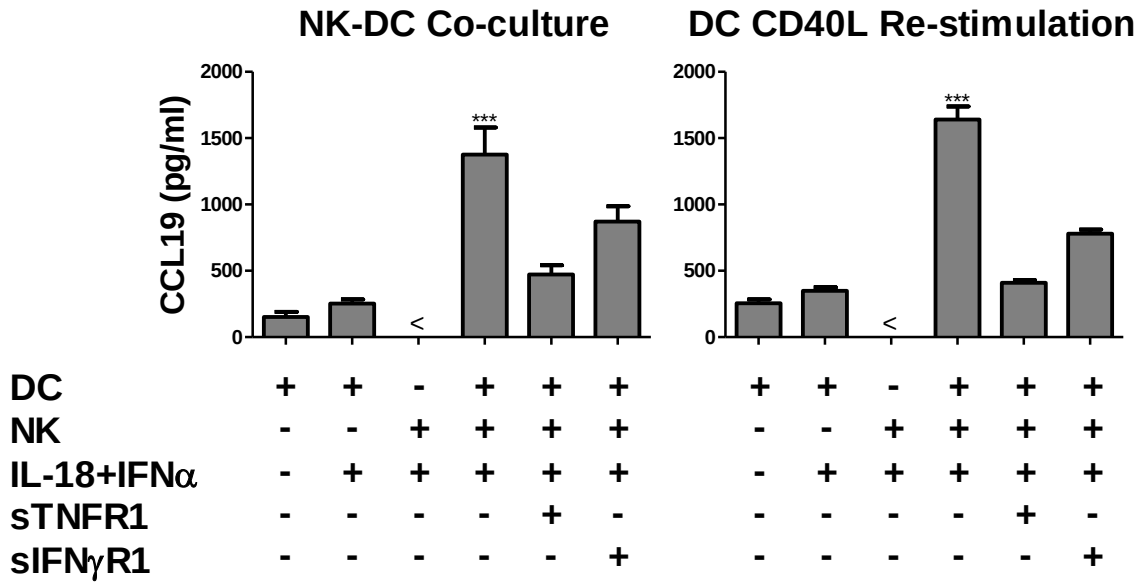

Figure 4.2. NK cell-induced DC production of CCL19 depends on TNFα and IFNγ.

CCL19 levels in 48 h culture supernatants (left) of untreated DCs or DCs exposed to IL-18/IFNα with or without autologous NK cells, in the additional presence or absence of soluble TNF (sTNFR1) or IFNγ (sIFNγR1) decoy receptors; or in 24 h culture supernatants (right) of NK cell-activated DCs alone after harvesting, washing, depletion of NK cells, and re-stimulation with CD40L. Data are recorded as the mean (± SD) of triplicate cultures. Data represent one of three independent experiments, which all yielded similar results. ***$p<0.001$ compared to all groups. < indicates levels were below the limit of detection of the assay.

4.4.3 NK cell-activated DCs efficiently recruit naïve T cells and promote their expansion and functional differentiation

Consistent with the significant enhancement of CCL19 secretion mediated by the interaction of IL-18-primed NK cells with DCs, supernatants from NK-DC co-cultures were highly effective at recruiting naïve $CD8^+$ T cells in transwell chemotaxis assays (Fig. 4.3A). Antibody blockade of CCL19's cognate receptor, CCR7, demonstrated that this enhanced migration of naïve $CD8^+$ T cells toward NK-DC supernatants was dependent on naïve T cell-expressed CCR7 (Fig. 4.3B).

Substantially-enhanced $CD8^+$ T cell numbers were observed in cultures of NK cell-activated DCs at day 7 following migration (Fig. 4.3C, left). Importantly, these T cells demonstrated robust proliferation as well as high expression of the CTL marker, granzyme B (Fig. 4.3C, middle and right), indicating that NK cell-activated DCs were capable of efficiently recruiting naïve $CD8^+$ T cells as well as inducing subsequent $CD8^+$ T cell expansion and activation toward effector capability.

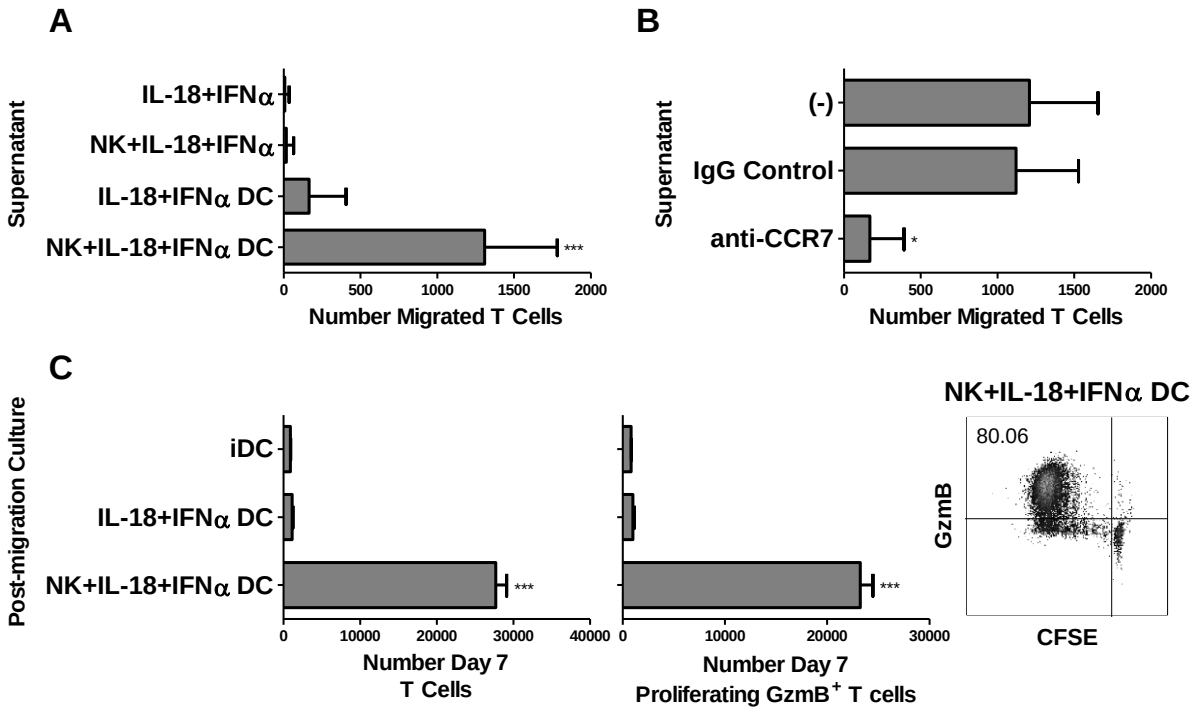

Figure 4.3. NK cell-activated DCs recruit naïve T cells and promote their expansion and functional differentiation. (A) Migration of naive $CD8^+$ T cells toward supernatants collected from 48 h cultures of IL-18/IFNα alone, NK cells treated with IL-18/IFNα, or DCs exposed to IL-18/IFNα with or without autologous NK cells. Data recorded as the mean (± SD) number of specific migrated T cells from 4 independent donors. (B) Migration of naive $CD8^+$ T cells, pretreated with or without anti-CCR7 blocking antibody, toward supernatants collected from 48 h cultures of DCs and autologous NK cells with IL-18/IFNα. Data recorded as the mean (± SD) number of specific migrated T cells from 4 independent donors. (C) Total number (left) or number of proliferated, granzyme B (GzmB) positive (middle) $CD8^+$ T cells in culture wells with DCs on day 7 after migration. Data recorded as the mean (± SD) number

of T cells in triplicate cultures. Representative CFSE and GzmB staining (right) in $CD8^+$ T cells after migration and 7 d co-culture with NK cell-activated DCs. ***p<0.001, *p<0.05 compared to all groups.

4.4.4 CCL19 production is induced in human tumor-associated lymph nodes by NK cell-targeting two-signal activation stimuli

To examine the relevance of the two-signal activation of NK cell helper activity in human lymphoid tissue for potential use in the treatment of cancer patients, we investigated the combined application of IL-18/IFNα to *ex vivo* LN explants from colorectal cancer patients. Treatment of these LNs with IL-18/IFNα augmented their expression of TNFα and IFNγ, and resulted in a marked enhancement of CCL19 expression within these LN tissues (Fig. 4.4), demonstrating the feasibility of applying NK cell-targeting, IL-18-based combinatorial adjuvants to promote T cell priming in human tumor-associated lymph nodes or peripheral lymph nodes targeted by cancer vaccines.

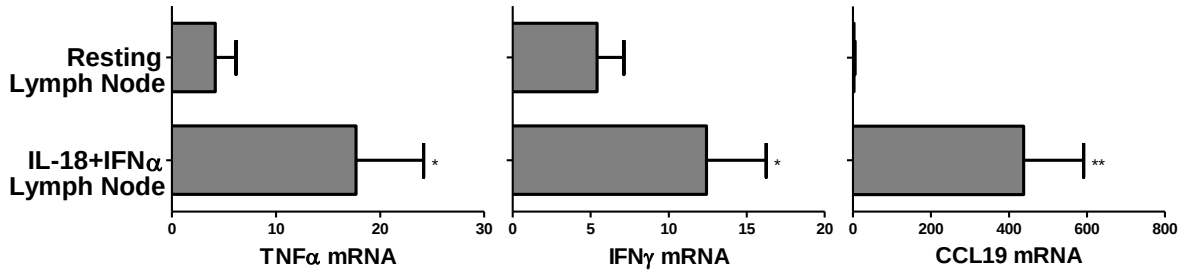

Figure 4.4. NK cell-activating stimuli drive CCL19 production in human tumor-associated lymph nodes.

Expression of TNFα, IFNγ, and CCL19 in lymph nodes obtained from colorectal cancer patients, cultured for 24 h in the absence or presence of IL-18/IFNα. Data are expressed as ratios between the expression of individual genes and HPRT1, and shown as the mean expression (± SD) of triplicate cultures of lymph node tissues. Data represent one of two independent experiments, which both yielded similar results. **p<0.01, *p<0.05 compared to other group.

4.5 DISCUSSION

These data describe a novel link between innate and adaptive immunity, demonstrating the helper role of NK cells in facilitating DC-mediated T cell priming by promoting DC CCL19 production. DC secretion of CCL19 is critical for the recruitment of naïve T cells to the T cell zones of secondary lymphoid tissue in both mice and humans *in vivo* [353, 354], as well as in promoting direct interactions between naïve T cells and DCs needed for the induction of adaptive immunity [352]. While DC production of CCL19 has been previously shown to be induced by direct DC infection [355] or by DC recognition of pathogen motifs [356], our current data demonstrate an alternative pathway of DC CCL19 production induced by NK cells, which specialize in detecting alternative forms of danger signals, such as oncogenic transformation [357]. Our findings are consistent with recent evidence *in vivo* indicating an important role for NK cells in driving the recruitment of both DCs and naïve T cells to the lymph node, necessary for the optimal development of protective T cell immune responses [358].

NK cells have been previously described to induce DC co-stimulatory molecule expression, IL-12 production, and lymph-node homing capacity, activities which are regulated in a two-signal mechanism importantly driven by IL-18 [190, 326]. The current data indicate that a similar two-signal mechanism also governs the NK cell ability to instruct DC production of CCL19, suggesting that NK cell-induced DC migration to LNs, attraction of naïve T cells, and priming responses are coordinately-regulated phenomena. Our data also indicate stable NK cell-induced DC production of CCL19 even after removal from the initial NK-DC interaction, suggesting that NK cell-mediated instruction of DC CCL19 secretion and the subsequent DC recruitment of naïve T cells for priming need not occur in the same compartment. Nevertheless, numerous reports have also indicated the ability of NK cells to traffic to LNs after activation in

mice *in vivo* [123, 359, 360], which may be driven in humans through NK cell acquisition of CCR7 via IL-18 in autologous settings [297] or via trogocytosis in the context of allogeneic therapy [361, 362], promoting coordinated NK cell, DC, and T cell interaction in secondary lymphoid tissue.

Given the expression of CCR7 and cognate CCL19 responsiveness across NK cells, DCs, and T cells, these data suggest the potential for CCL19 initially elicited by NK-DC interaction to participate in a potent feed-forward accumulation of all three cell types in the development of robust priming responses. Indeed, DCs activated in the presence of IFNγ, a key NK cell-provided factor and an important inducer of DC CCL19 production shown in this study, have been demonstrated to reciprocally enhance the CCL19 responsiveness and activation of NK cells [363], likely recruiting additional NK cells and providing for an amplifying cycle of DC activation. Thus, the NK-DC collaborative mechanism described here suggests the ability to develop significant immunity in response to the detection of relatively small pathogenic or oncogenic stimuli.

The amplification potential of this NK-DC interaction may present an attractive therapeutic target to augment immune activation for the treatment of cancer and chronic infections. Our data demonstrate that effective combinations of helper NK cell-activating factors can induce CCL19 expression in tumor-associated LNs from colorectal cancer patients, suggesting the possibility for the therapeutic enhancement of chemokine-driven T cell priming in the human cancer setting, either by targeting tumor tissues, tumor-draining LNs, or LNs draining sites of cancer vaccination. Indeed, several studies have shown the beneficial impact of CCL19 in therapeutic tumor models [364-367], which have been supported by correlations between CCL19 and prolonged survival in cancer patients [25].

Increasing evidence also suggests that CCL19-driven anti-tumor immune responses directly within the tumor environment may be important for protective immunity, including within tertiary lymphoid structures (TLS) containing close interactions between mature DCs and naïve T cells [21]. Such TLS and intratumoral tissues bearing lymphoid organ features, such as high endothelial venules, have been shown to correlate with both CCL19 expression and favorable clinical outcome in patients with non-small-cell lung cancer and breast cancer [368-370]. Likewise, in renal cell carcinoma, CCL19 has been shown to co-localize in tumor regions bearing clusters of mature DCs and proliferating $CCR7^+$ T cells [371], with high tumor infiltration of $CCR7^+$ T cells found to predict prolonged survival in advanced colorectal cancer [372]. These findings further support the strong therapeutic rationale for intratumoral activation of CCL19 production induced by NK-DC interaction.

In summary, these data indicate a novel helper NK cell-driven mechanism for promoting DC-mediated T cell priming through the key regulation of CCL19 interactions, and support the therapeutic application of NK cell-targeting, IL-18-based combinatorial adjuvants in cancer patients to enhance anti-tumor immunity.

5.0 TUMOR-ASSOCIATED MDSCS INDUCE CD8$^+$ T CELL SUPPRESSION AND CD4$^+$ T CELL DIFFERENTATION TOWARD TH17 IMMUNITY

Adapted from:

Induction and stability of human Th17 cells require endogenous NOS2 and cGMP-dependent NO signaling

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These data are reported in the *Journal of Experimental Medicine* (2013, in press). J. L. Wong contributed to designing the study; developing methodology and acquiring data (including data shown in Fig. 5.4, A and D-E); analyzing and interpreting all data in the study; and writing, reviewing, and revising the manuscript.

5.1 ABSTRACT

Myeloid-derived suppressor cells (MDSCs) are central players in defining the immune environment in cancer. We observed that while human cancer-associated $CD11b^{+}CD33^{+}CD34^{+}HLA\text{-}DR^{low}$ MDSCs suppress $CD8^{+}$ T cell responses, MDSCs interacting with $CD4^{+}$ T cells secrete IL-1β, IL-6, IL-23, and nitric oxide (NO), promoting the induction of $ROR\gamma t^{+}$ Th17 cells expressing endogenous nitric oxide synthase-2 (NOS2). While high concentrations of exogenous NO indiscriminately suppress the proliferation and differentiation of Th1, Th2, and Th17 cells, physiologic NO concentrations produced by cancer-associated MDSCs and by Th17 cells themselves are essential for the development and stability of human $ROR\gamma t^{+}IL\text{-}23R^{+}IL\text{-}17^{+}$ Th17 cells, consistent with a positive *in vivo* correlation between IL-17 and NOS2 observed in the malignant ascites from ovarian cancer patients. The development of Th17 cells from naive-, memory-, or tumor-infiltrating $CD4^{+}$ T cells, driven either by IL-1β/IL-6/IL-23/NO-producing MDSCs or by recombinant cytokines (IL-1β/IL-6/IL-23), critically depends on endogenous NOS2 induced in $CD4^{+}$ T cells, and on the canonical cGMP/cGK pathway of NO signaling. Inhibition of NOS2 or cGMP/cGK signaling abolishes both the *de novo* induction of Th17 cells as well as IL-17 production by established Th17 cells isolated from ovarian cancer patients, providing new targets to manipulate Th17-associated immunity in cancer and inflammatory diseases.

5.2 INTRODUCTION

Human cancer-associated myeloid-derived suppressor cells (MDSCs), characterized by a $CD11b^{+}CD33^{+}HLA\text{-}DR^{low/neg}$ phenotype consisting of $CD14^{+}$ monocytic [234, 245, 249, 373, 374] and/or $CD15^{+}$ granulocytic [251, 375, 376] subsets (reviewed in [235, 377]), accumulate in most cancer patients in response to pro-inflammatory mediators. Using a variety of suppressive mechanisms [228, 234, 377-379], cancer-associated MDSCs have been linked to the induction of T cell dysfunction through the production and/or metabolism of TGF-β [380, 381], ROS [252, 382, 383], NOS2/NO [265], L-arginine [266, 375, 384, 385], peroxynitrites [384, 386], IL-10 [267], and PGE_2 [234], and may represent the major population responsible for the induction of antigen-specific $CD8^{+}$ T cell tolerance in cancer. However, their exact role within the network of specific $CD4^{+}$ T cell subsets, encompassing various populations with distinct cellular and immunological functions, remains unclear.

Nitric oxide (NO; a product of nitrite reduction or the NO synthases NOS1, NOS2 and NOS3; [387]), is a pleiotropic regulator of neurotransmission, inflammation, and autoimmunity [387-390] implicated both in cancer progression and its immune-mediated elimination [387, 391-393]. In different mouse models, NO has been paradoxically shown to both promote inflammation [394-398] as well as suppress autoimmune tissue damage through non-selective suppression of immune cell activation [389, 399], especially at high concentrations [400-402]. While previous studies demonstrated a positive impact of NO on the induction of Th1 cells [403] and $FoxP3^{+}$ regulatory T cells (T_{reg}) [404] in murine models, the regulation and function of the NO synthase (NOS)/NO system have shown profound differences between mice and humans [405-407], complicating the translation of these findings from mouse models to human disease.

In cancer, NOS2-derived NO plays both cytotoxic and immunoregulatory functions [389]. It can exert distinct effects on different subsets of tumor-infiltrating T cells (TILs), capable of blocking the development of cytotoxic T lymphocytes (CTLs) [408], suppressing Th1 and Th2 cytokine production, and modulating the development of $FoxP3^+$ T_{regs} [409, 410]. NOS2-driven NO production has been shown to be a prominent feature of cancer-associated myeloid-derived suppressor cells (MDSCs) [253, 265, 382], but the significance of this production to the differentiation of specific T cell subsets, particularly in the human cancer setting, is unknown.

Production of NO in chronic inflammation is supported by IFN-γ and IL-17 [265, 411], the cytokines produced by human Th17 cells [151, 412-415]. Human Th17 cells secrete varying levels of IFN-γ [151, 413-417] and have been implicated both in tumor surveillance and tumor progression [416-418]. Induction of Th17 cells typically involves IL-1β, IL-6, and IL-23 [151, 412-415, 419-421], with the additional involvement of TGF-β in most mouse models [412, 420-423], but not in the human system [414, 415]. IL-1β, IL-6, and IL-23 production by monocytes and dendritic cells (DCs), and the resulting development of human Th17 cells, can be induced by bacterial products, such as LPS or peptidoglycan [151, 413, 414]. However, the mechanisms driving Th17 responses in non-infectious settings, such as autoimmunity or cancer, remain unclear.

Here, we report that the development of human Th17 cells from naïve, effector, and memory $CD4^+$ T cell precursors induced by the previously-identified Th17-driving cytokines (IL-1β, IL-6, and IL-23) or by IL-1β/IL-6/IL-23-producing MDSCs, is promoted by exogenous NO (or NO produced by human MDSCs) and critically depends on the induction of endogenous NOS2 in differentiating $CD4^+$ T cells. The ability of inhibitors of NOS and the canonical

cGMP/cGK signaling pathway of NO to prevent the *de novo* induction of Th17 cells and suppress the function of pre-existing Th17 cells from cancer patients provides new targets for the modulation of Th17-associated immune events in cancer, inflammatory, and infectious diseases.

5.3 MATERIALS AND METHODS

Media and reagents. Cells were cultured in IMDM medium (Invitrogen) with 10% FCS (Gemini). DETA-NONOate was purchased from Cayman Chemical and used at the concentration of 25 μM, unless otherwise specified. General NOS inhibitors L-NMMA (Sigma) and ADMA (Sigma) and the NOS2-specific inhibitor 1400W (Sigma) were used at the concentrations of, respectively, 100 μM, 200 μM, and 200 nM, unless indicated otherwise. Arginase inhibitor nor-NOHA (Cayman Chemical) was used at 200 μM, IDO inhibitor 1-Methyl-D-tryptophan (Sigma) was used at 1 mM, neutralizing α-IL-10 mAb (R&D; clone 25209) was used at 1.0 μg/ml, COX2 inhibitor celecoxib (Biovision) was used at 20 μM, c-GMP analogue Br-cGMP (Sigma) was used at 100 μM, and cGMP inhibitor ODQ was used at 10 μM. Th1, Th17, and T_{reg}-driving cytokines were used at the following concentrations (unless stated otherwise): IL-1β (20 ng/ml; Miltenyi Biotec), IL-6 (50 ng/ml; ThermoFisher Scientific), IL-23 (10 ng/ml; R&D), TGF-β_1 (5 ng/ml; R&D), and 9-cis retinoic acid (10 nM; Sigma). CFSE (Invitrogen) labeling kit to monitor cell proliferation was used according to the manufacturer's protocol. CD3/CD28 stimulation was accomplished with anti-CD3/CD28 human or mouse T cell-activator Dynabeads (at 2 μl/ml; Invitrogen). Soluble (s)CD40L was used at 1 μg/ml in combination with 1 μg/ml of Enhancer for Ligands (Enzo Life Sciences). Nitrite formed by the

spontaneous oxidation of NO under physiological conditions in cell culture supernatants was detected with the Griess reagent kit (Invitrogen) according to the manufacturer's protocol.

Isolation of peripheral blood naïve and memory human $CD4^+$ and $CD8^+$ T cells and mouse splenic $CD4^+$ T cells. Human PBMCs were isolated from buffy coats provided by the Central Blood Bank of Pittsburgh, PA. T cells were isolated from PBMCs by negative selection using $CD4^+$ and $CD8^+$ T cell enrichment cocktails (Stem Cell Tech) in combination with either anti-CD45RO or CD45RA depletion antibodies, resulting in a >95% pure $CD3^+$ population of uniform $CD4^+/8^+CD45RA^{+/-}CD45RO^{+/-}$ cells. Allogeneic combinations of T cells and MDSCs were used to allow testing of the impact of tumor-derived MDSCs (or control blood-isolated $CD11b^+$ cells) on the differentiation of healthy donor naïve or memory blood-isolated T cells. Mouse naïve and memory $CD4^+$ T cells were isolated from the spleens of C57BL/6 (B6) mice using naïve and memory $CD4^+$ T cell isolation kits (Miltenyi Biotec). Six- to 8-week-old wild-type B6 mice were purchased from Taconic (Germantown, NY). Specific pathogen-free mice were used in all experiments and housed in pathogen-free conditions at Children's Hospital of Pittsburgh, Pennsylvania. All of the animal studies were conducted with the approval of the University of Pittsburgh Institutional Animal Care and Use Committee.

Isolation of MDSCs and OvCa-infiltrating $CD4^+$ T cells (TILs). Human OvCa ascites cells were obtained intraoperatively from previously-untreated patients with primarily advanced stage III or IV epithelial OvCa, after obtaining written informed consent. The nature and possible consequences of the studies were explained. All specimens were provided under protocols approved by the University of Pittsburgh or Roswell Park Cancer Institute Institutional Review Boards. Human OvCa ascites obtained from the University of Pittsburgh (IRB0406147) were used in the isolation of cancer-associated $CD11b^+$ cells (MDSCs) and subsequent isolation of

$CD4^+$ TILs. The median age of patients was 56 years old (range 39-69 years old). Twelve patients were Causcasian and one patient was African-American. The majority of patients were FIGO Stage IIIC, one patient was Stage IIIA, and one patient was Stage IIA. Tumor histology was serous in 9 cases (69.2%), clear cell in 2 cases (15.4%), mucinous in 1 case (7.7%), and mixed histology in 1 case (7.7%). Human OvCa ascites obtained from the Roswell Park Cancer Institute (CIC02-15) were used in the isolation of bulk OvCa primary cells and their CD3/CD28-driven expansion for 7 days in culture. The median age of patients was 64 (range 50-85). Nine patients were Causcasian and one was Hispanic. The majority of patients were FIGO Stage IIIC, three patients were Stage IV. Tumor histology was serous in 7 cases (70%), papillary serous in 2 cases (20.0%), and mixed histology in 1 case (10%).

OvCa primary cells were harvested by centrifugation. $CD11b^+$ cells (i.e. MDSCs) were obtained after centrifugation of OvCa ascites, followed by red blood cell lysis and positive magnetic selection of $CD11b^+$ cells (CD11b EasySep Isolation kit; Stem Cell Tech). The isolated cells were >95% $CD11b^+$ and uniformly expressed the $CD11b^+CD33^+CD34^+$ MDSC phenotype [234]. $CD4^+$ T cells (TILs) were obtained after positive magnetic selection of $CD11b^+$ cells followed by negative selection using the $CD4^+$ T cell enrichment cocktail (Stem Cell Tech). Control $CD11b^+$ cells were isolated from healthy donor buffy coats, using the same method.

Th17 cell generation. T cells were stimulated with anti-CD3/CD28 Dynabeads (2.0 µl/ml; Invitrogen) in the presence or absence of allogeneic OvCa-isolated MDSCs or control $CD11b^+$ cells, pretreated or not with inhibitors, and/or in the presence of the Th17-inducing cytokine cocktail: IL-1β (20 ng/ml), IL-23 (10 ng/ml), IL-6 (50 ng/ml), and/or TGF-β_1 (10 ng/ml). All experiments used $1x10^5$ T cells per well at a concentration of $5x10^5$ cells/ml. All experiments in this study used the 1:4 ratio of MDSC (or control $CD11b^+$ cells) to T cells,

determined to be optimal based on our preliminary experiments (which tested the MDSC:T cell ratios of 1:1, 1:2, 1:4, and 1:8). As an alternative to stimulation with anti-CD3/CD28 beads, T cells were stimulated with mature DCs (mDCs) [monocytes were isolated by positive magnetic selection using the EasySep $CD14^+$ isolation kit (Stem Cell Tech) and cultured for 6 days in 24-well plates (BD) in the presence of rhuGM-CSF and IL-4 (both 1000 U/ml; gifts from Schering Plough) and afterwards matured for 48 h with TNF-α], with a DC:T cell ratio of 1:10. On day 4-6, expanded T cells were analyzed for the expression of Th17-associated factors (mRNA expression) and cytokine secretion (ELISA). On day 7-8, expanded T cells were assayed for intracellular NOS2 and NO, and were further re-stimulated for intracellular cytokine staining (all as further described below). The purity of anti-CD3/CD28–activated T cell cultures increased from an initial >95% to over 99% by the time of analysis, as determined by flow cytometry. Note that, consistent with the key role of IL-1β and IL-6, and the negative role of TGF-β_1, in the induction of human Th17 cells [414, 415], and a similar synergy between IL-1β and other Stat3 activators [424], the combination of IL-1β with IL-6 and/or IL-23 was sufficient for the optimal induction of IL-17A production, with TGF-β_1 having a negative effect (Fig. 5.4G).

ELISA. ELISA analysis was performed for IL-17A (R&D) and IFN-γ secretion by day 5 expanded T cells in culture [or by day 7-8 expanded T cells washed and re-plated at $1x10^6$ cells/ml and re-stimulated with anti-CD3/CD28 beads for 24-48 h]. ELISA analysis of IL-23 production by OvCa ascites-isolated MDSCs or control blood-isolated $CD11b^+$ cells was performed after 24 h stimulation with CD40L-expressing J558 cells [or sCD40L (Enzo Life Sciences)] or $CD4^+$ T cells. 24 h-conditioned medium from OvCa ascites-isolated MDSCs or control blood-isolated $CD11b^+$ cells were analyzed for IL-6, IL-10, IL-23, IL-1b, and TGF-β_1 by sandwich ELISA (R&D).

Flow cytometry. Two- and three-color cell surface and intracellular immunostaining analyses were performed using an Accuri C6 flow cytometer. OvCa ascites-isolated cells were stained with the antibodies CD11b-FITC, CD33-APC, CD34-PE/Cy7, HLA-DR-FITC, CD14-PE, CD80-FITC, CD83-PE, CD15-PE, and CD8-FITC (BD and eBioscience) (see [234] for full phenotype). IL-23R was detected with IL-23R-FITC mAb (R&D). Rat $IgG_{2\alpha}$-PE, IgG_1-FITC, IgG_1-APC, and IgG_1-PE/Cy7 isotype controls, and the rat $IgG_{2\alpha}$-FITC isotype control, were from BD PharMingen.

Intracellular staining. Cells were harvested, fixed, and permeabilized using the Foxp3 Fix/Perm Buffer Set solution (eBioscience). For intracellular cytokine production only, T cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich), and after 4 h, brefeldin A (10 μg/ml) was added for an additional 4-10 h prior to staining. The following antibodies were used: IFN-γ-FITC and IL-17A-PE (eBio64DEC17; eBioscience), Foxp3-Alexa Fluor 488 (BioLegend), CD3 [unlabeled monoclonal mouse anti-human CD3 (eBioscience) followed by secondary goat anti-mouse IgG $F(ab')_2$-Alexa Fluor 647 (Cell Signaling Technology)], and NOS2 [unlabeled polyclonal rabbit anti-human NOS2 (Millipore) followed by secondary goat anti-rabbit IgG $F(ab')_2$-Alexa Fluor 488 (Cell Signaling Technology)]. Stainings (including for both primary and secondary antibodies, where appropriate) were performed at room temperature for 30 min, then washed and resuspended in FACS buffer prior to analysis. For intracellular NO detection, DAF-FM diacetate (Molecular Probes) was used, which passively diffuses across cell membranes and is deacetylated by intracellular esterases to membrane-impermeant DAF-FM, which then reacts with NO to form a fluorescent benzotriazole with excitation/emission maxima of 495/515 nm [425]. DAF-FM diacetate was loaded at 10 μM for 2 h at 37°C.

Confocal microscopy. T cells were harvested and directly centrifuged onto 12 mm diameter circular glass coverslips (Propper) coated for 1 h at 37°C with 0.005% human fibronectin (Sigma) in PBS in 24 well plates. The coverslips were then incubated in 4% paraformaldehyde for 15 min, washed with PBS, and incubated for 1 h at room temperature in staining buffer containing 0.3% Triton X-100 (Sigma), 5% goat serum (Life Technologies), and 1% BSA (Fisher Scientific) in PBS. The slides were then incubated for 3 h at room temperature with staining buffer containing unlabeled primary antibodies for NOS2 (Millipore) and CD3 (eBioscience), washed with PBS, and incubated for 30 min at room temperature with staining buffer containing the secondary antibodies anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647 (Cell Signaling Technology). Coverslips were washed with PBS and mounted on SuperFrost Plus Slides (Thermo Scientific) using ProLong Gold antifade reagent (Invitrogen). Confocal analyses were conducted using a LEICA TCS SL DMRE Microsystem.

Taqman analysis of mRNA expression. mRNA expression was analyzed in day 7-8 anti-CD3/CD28-expanded OvCa primary cell cultures, and in day 4-6 expanded $CD4^+$ TILs and naive and memory $CD4^+$ T cells. Taqman analysis was performed on the StepOne Plus System (Applied Biosystems) using Taqman-recommended inventoried or made-to-order assays (Gene IDs: il17a:Hs00174383, il17f:Hs01028648, il2rα:Hs00907779, il23r:Hs00332759, nos2:Hs01075527, rorc:Hs01076112, tbet:Hs00203436, gata3:NM_002051, foxp3:Hs00203958). The expression of each gene was normalized to HPRT1 and expressed as relative expression, i.e. fold increase ($2^{-\Delta CT}$), where $\Delta CT = CT_{(\text{Target gene})} - CT_{(\text{HPRT1})}$.

Statistical analysis. The figures demonstrating key phenomena and critical mechanisms involve aggregate data from multiple patients and healthy donors (expressed as means ± SD; the donor numbers are provided in the legends for individual figure panels). Data from

representative experiments (typically used in the studies comparing different reagents or different concentrations) were obtained from replicate cultures (means ± SD; numbers of replicates provided in figure legends) with each experiment confirmed in additional independent experiments with cells from different donors, as indicated in the figure legends. All data were evaluated using GraphPad Prism Version 5 software and analyzed using Student's t test (two-tailed) and 1-way and 2-way analysis of variance, where appropriate, with $P<0.05$ considered as significant ($P<0.05$ marked *; $P<0.01$ marked **; $P<0.001$ marked ***). A linear correlation between two continuous variables was tested with the r^2 coefficient of determination.

5.4 RESULTS

5.4.1 MDSCs suppress CD8$^+$ T cells while MDSC-associated NOS2/NO promote the Th17 phenotype in ovarian cancer patient TILs and naïve and memory CD4$^+$ T cells

While tumor-associated MDSCs have been described to be potent suppressors of T cell responses [228], the high frequencies of Th17 cells observed in ovarian cancer (OvCa; [416, 417]) and our observations that the OvCa environment is a potent inducer of Th17 responses [417] prompted us to test the effect of cancer-associated MDSCs on CD8$^+$ and CD4$^+$ T cells, and in particular, the role of MDSCs in the development of human Th17 immunity. Interestingly, while OvCa patient-isolated MDSCs, expressing the typical CD11b$^+$CD33$^+$HLA-DR$^{-/low}$CD80$^-$ phenotype [26, 228] (*data not shown*, see [234] for complete MDSC phenotype), strongly suppressed the CD3/CD28-induced proliferation of naive CD8$^+$ T cells, they did not suppress the proliferation of naive CD4$^+$ T cells (Fig. 5.1).

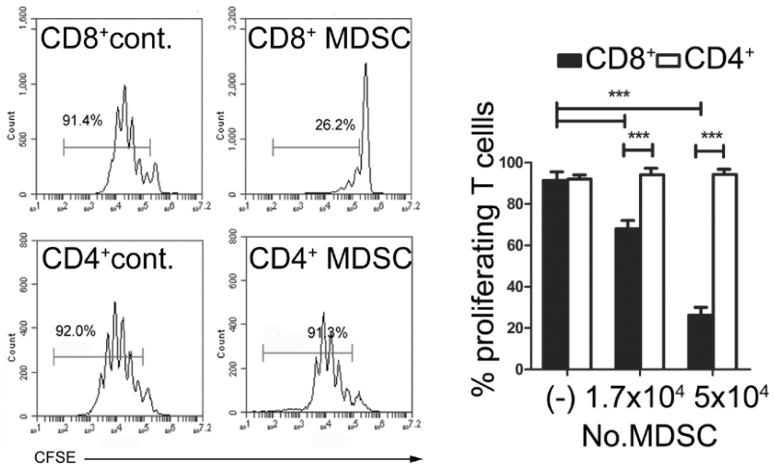

Figure 5.1. Ovarian cancer-associated MDSCs suppress $CD8^+$ but not $CD4^+$ T cell proliferation.

Percentage of proliferating naïve $CD8^+$ and $CD4^+$ T cells following 4 d activation with anti-CD3/CD28 beads in the absence or presence of OvCa ascites-isolated $CD11b^+$ cells (i.e. MDSCs) or control blood-isolated $CD11b^+$ cells, measured by CFSE dilution in representative cultures (left) and across triplicate cultures (right). ***$p<0.001$.

Instead, these MDSCs selectively enhanced the expression of Rorc (encoding RORγt) and IL-17A (Fig. 5.2A) and the production of IL-17A protein (Fig. 5.2, B and C) by $CD4^+$ T cells, activated by anti-CD3/CD28 antibodies or allogeneic DCs (Fig. 5.2C), with no impact on the Th1 marker, T-bet, and an inhibitory impact on the T_{reg} marker, FoxP3 (Fig. 5.2A). Unexpectedly, while inhibition of other MDSC products, including IL-10, IDO, and arginase, did not impact the induction of Th17 cells (Fig. 5.2, D and E), the inhibition of NOS, the key synthesizing enzyme for the nominally-suppressive MDSC product NO [236, 239], dose-dependently inhibited the ability of MDSCs to enhance IL-17A production (Fig. 5.2, D-F) and Rorc expression (Fig. 5.2G) in activated $CD4^+$ T cells. In addition, the Th17-promoting effect of MDSCs was also reduced by COX2 inhibition (Fig. 5.2, D-F), the factor needed for the optimal MDSC expression of NOS2 [234].

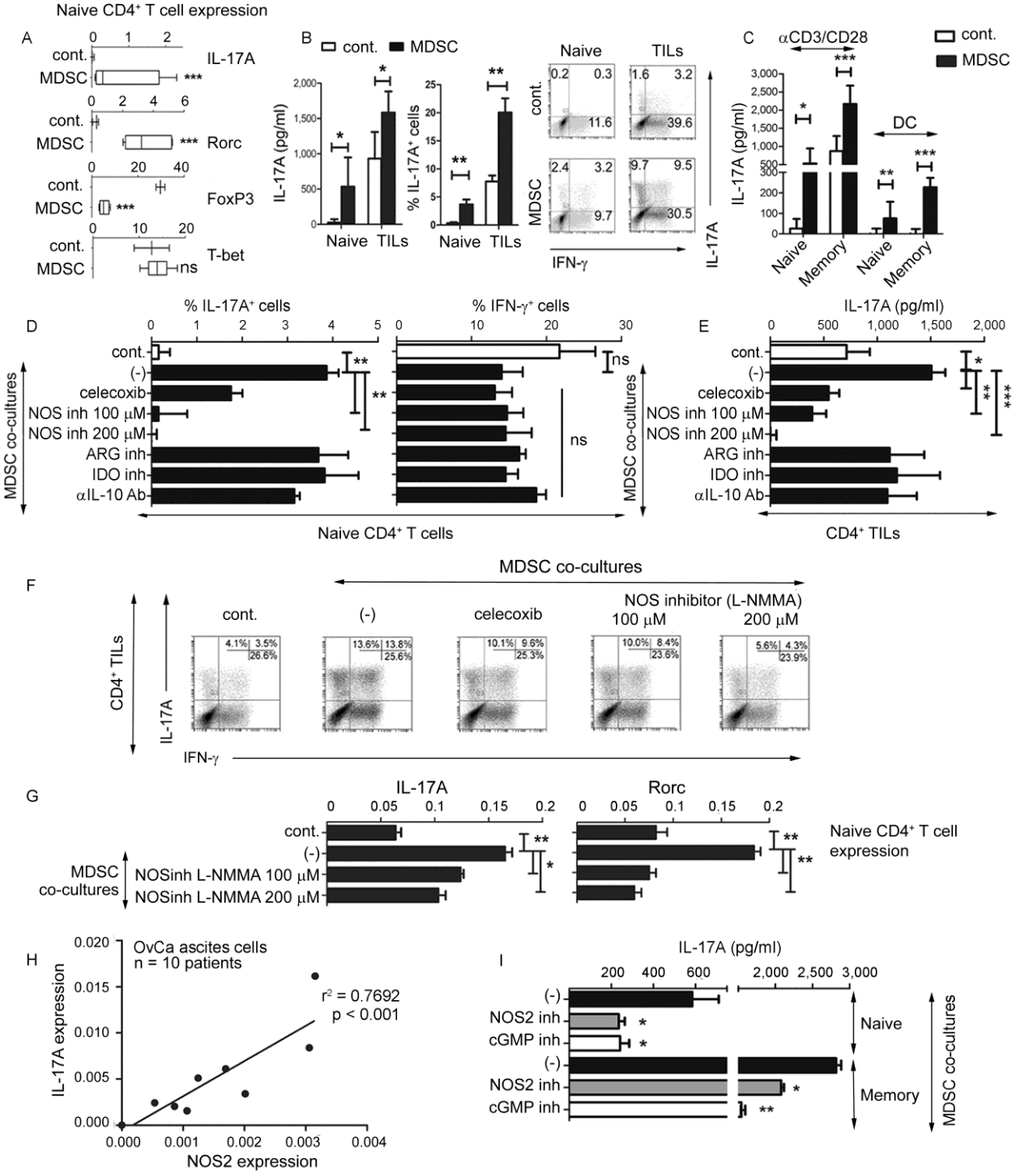

Figure 5.2. Key role of NOS2 and canonical cGMP-mediated NO signaling in the MDSC-promoted Th17 differentiation of TILs from ovarian cancer patients and human naïve and memory $CD4^+$ Th cells.

(A) Induction of IL-17A, Rorc (encoding RORγt), FoxP3 (indicating *de novo* differentiation of $FoxP3^+$ T_{regs} from naive precursors), and T-bet gene expression in anti-CD3/CD28-expanded naive $CD4^+$ T cells by tumor–isolated

MDSCs (mean ± SD from 6 patients), as compared to control $CD11b^+$ cells (mean ± SD from 3 healthy donors). (B) IL-17A production levels and percentages of $IL\text{-}17A^+$ cells (mean ± SD from n donors), and representative intracellular staining (IL-17A vs. IFN-γ, right) in naive $CD4^+$ T cells (n=6 healthy donors) or OvCa-infiltrating $CD4^+$ TILs (n=3 patients) stimulated with anti-CD3/CD28 antibodies in the presence of cancer-isolated MDSCs or control $CD11b^+$ cells. (C) IL-17A production by naïve vs. memory $CD4^+$ T cells (mean ± SD from n=4 healthy donors) stimulated with either anti-CD3/CD28 antibodies or TNF-α-matured allogeneic DCs in the presence of MDSCs or control $CD11b^+$ cells. (D-E) Percentage of $IL\text{-}17A^+$ or $IFN\text{-}\gamma^+$ $CD4^+$ T cells (D) and IL-17A secretion (E) in anti-CD3/CD28/MDSC-expanded naïve $CD4^+$ T cells (D) and $CD4^+$ TILs (E), by specific inhibitors of NOS (L-NMMA), COX2 (celecoxib), IDO, ARG, or IL-10. The data (mean ± SD) from one representative experiment (performed in replicates: D, triplicate cultures; E, quadruplicate cultures). The results were confirmed in 3 independent experiments using different patients/healthy donors. (F) Representative staining of $IL\text{-}17A^+$ or $IFN\text{-}\gamma^+$ $CD4^+$ T cells in co-cultures of anti-CD3/CD28-expanded $CD4^+$ TILs and MDSCs by specific inhibitors of COX2 (celecoxib) and NOS (L-NMMA). The results were confirmed in 3 independent experiments using different patients. (G) Relative gene expression of IL-17A and Rorc, induced in anti-CD3/CD28-expanded naive $CD4^+$ T cells cultured in the presence of MDSCs, by a specific inhibitor of NOS (L-NMMA). The graphs present the mean ± SD from one representative experiment (triplicate cultures) of two (using different patients/healthy donors). (H) Relative gene expression of NOS2 and IL-17A in 7 d *ex vivo* anti-CD3/CD28-expanded cultures of OvCa ascites cells from 10 OvCa patients (n=10, $r^2=0.7692$, $p<0.001$). (I) IL-17A production in anti-CD3/CD28-expanded cultures of naïve or memory $CD4^+$ T cells in the presence of MDSCs, with or without specific inhibitors of NOS2 (1400W) or cGMP (ODQ). Data (mean ± SD) from one representative experiment (triplicate cultures). The results were confirmed in 3 independent experiments using different patients/healthy donors. ns: $P>0.05$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

In accordance with the key role of MDSC-associated NOS2 in the induction of Th17 responses in cancer patients *in vivo,* we observed that the levels of NOS2 expression in the ascites cells from OvCa patients positively correlated with the ability of these cells to produce IL-17A after short-term *ex vivo* stimulation (Fig. 5.2H). Moreover, we observed that the Th17-promoting effects of MDSCs could be prevented both by the selective inhibition of NOS2 activity as well as

the inhibition of cGMP function (Fig. 5.2I), further demonstrating that human Th17 responses critically depend on NOS2 and the canonical cGMP/cGK-mediated signaling pathway associated with the physiologic NO concentrations produced by human cancer-isolated MDSCs (Fig. 5.3A).

While MDSCs produced all the previously-identified Th17-driving cytokines (Fig. 5.3B) either spontaneously (IL-1β, IL-6, and TGF-β_1) or after their stimulation (IL-23) with CD40L (the $CD4^+$ T cell-expressed APC-activating factor [426]; similar data was obtained with CD40L-expressing J558 cells or $CD4^+$ T cells, *data not shown*), the advantage of MDSCs in driving the Th17 phenotype (*data not shown*) was particularly pronounced in the case of naïve, as compared to memory, Th cells (Fig. 5.2C). Human naïve Th cells, compared to their memory counterparts, were previously shown to be less sensitive to the induction of the Th17 phenotype by recombinant cytokines [151], indicating that such an additional signal, which can be promoted by MDSCs, is essential for the *de novo* induction of Th17 cells.

Whereas, consistent with previous reports [400, 402], high doses of exogenous NO (higher than 100 μM; known to have cytostatic function [389]) non-selectively blocked $CD4^+$ T cell differentiation (Fig. 5.3C) and their proliferation (Fig. 5.3D) in all conditions tested (Th17-, Th1-, or T_{reg}-driving conditions), the application of lower, standard cell-signaling doses [390, 427] of NO donor (10-25 μM; comparable to the MDSC-produced NO levels; see Fig. 5.3A) did not affect $CD4^+$ T cell proliferation. Instead, these lower doses of NO selectively enhanced IL-17A production without affecting IFN-γ production (Fig. 5.3C), further confirming the ability of physiologic NO concentrations to selectively support human Th17 development.

Exogenous NO strongly enhanced the induction of Th17 cells driven by recombinant cytokines (Fig. 5.3, C and E-F), indicating its direct impact on T cells rather than its modulation of MDSC functions. NO induced IL-17A expression (Fig. 5.3E) and secretion (Fig. 5.3F) by

both naive and memory CD4$^+$ T cells and enhanced their expression of Rorc (but not GATA3, FoxP3, or T-bet; Fig. 5.3E and *data not shown*). The distinct Th17-promoting effect of exogenous NO was evident even in the absence of Th17-driving cytokines (IL-1β, IL-6, and IL-23; Fig. 5.3, C and E-F), indicating that NO is a direct inducer of Th17 differentiation, rather than a mere enhancer of the effects of Th17-inducing cytokines.

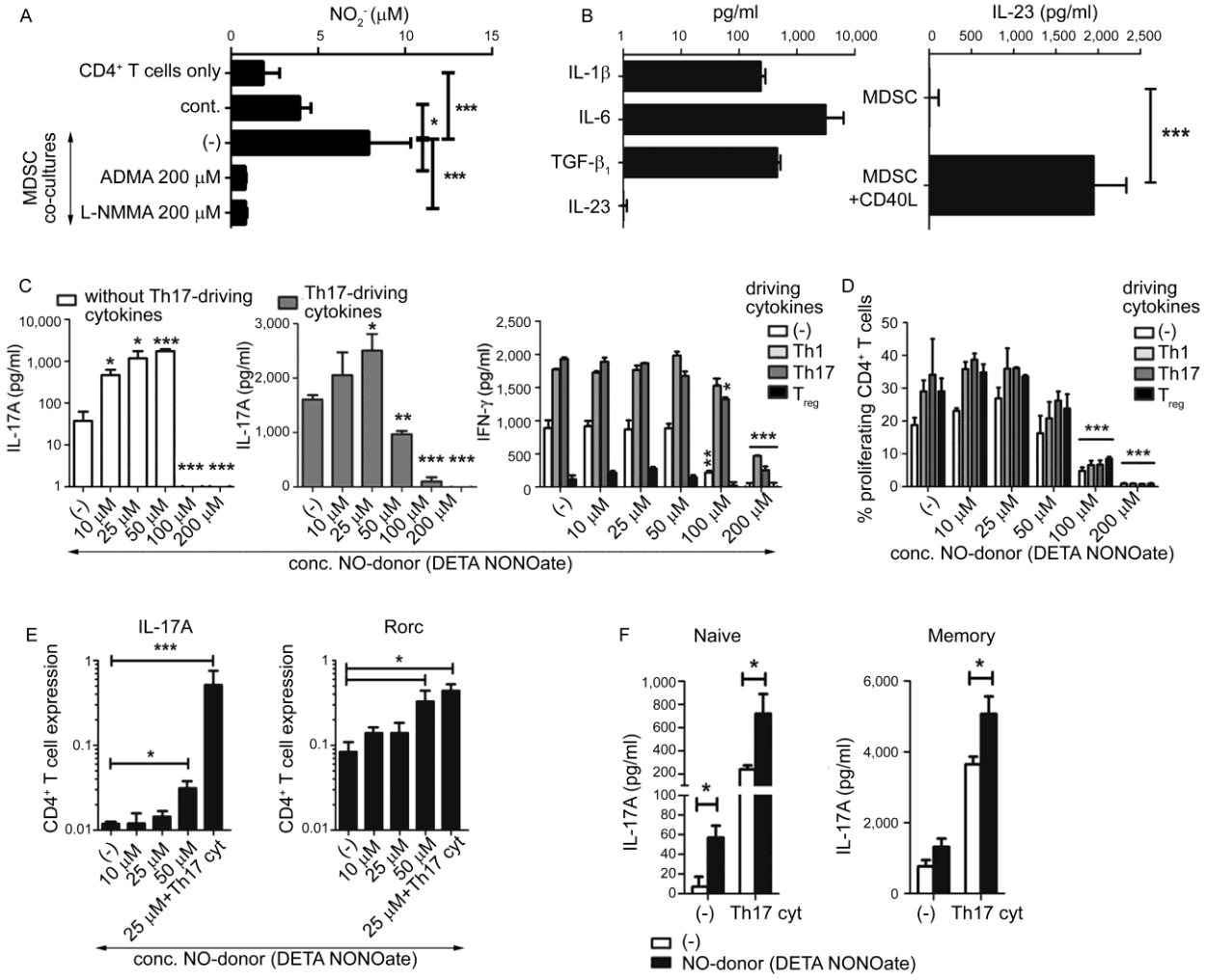

Figure 5.3. Exogenous NO supports the cytokine-driven induction of Th17 function in memory Th cells and promotes the *de novo* induction of Th17 cells from naïve precursors.

(A) NO_2^- levels (mean ± SD from 4 patients) in co-cultures of CD4$^+$ TILs with tumor-isolated MDSCs (as compared to blood CD11b$^+$ cells) and in the presence of NOS inhibitors ADMA and L-NMMA. (B) Expression of IL-1β, IL-6, TGF-β_1 (spontaneous expression, *left*), and IL-23 (stimulation with CD40L, *right*) in MDSCs. Data (mean ± SD)

from 3 experiments involving MDSCs from 3 different patients. (C) Induction of IL-17A or IFN-γ production by anti-CD3/CD28-stimulated bulk $CD4^+$ T cells from healthy donors, cultured in the absence or presence of Th1 (200U/ml rhIL-12, 200ng/ml αIL-4-Ab), Th17 (20ng/ml rhIL-1β, 50ng/ml rhIL-6, 10ng/ml rhIL-23), and T_{reg} (5ng/ml TGF-β_1, 10 nM 9-cis retinoic acid)-driving cytokines, and physiologic concentrations of exogenous NO donor (DETA-NONOate). IL-17A was undetectable in Th1- and T_{reg}-driving conditions. Percentage of $FoxP3^+$ cells in control cultures were (-): 10.4%, Th1: 12.7%, Th17: 5.2%, and T_{reg}: 41.2%. The graphs present the mean ± SD from one representative experiment (triplicate cultures) of two (healthy donors). (D) Suppression of $CD4^+$ T cells differentiating in Th1-, T_{reg}-, and Th17-driving conditions by high concentrations (>100 μM) of DETA-NONOate. Proliferation of CFSE-labeled anti-CD3/CD28-stimulated bulk $CD4^+$ T cells cultured in the absence or presence of Th1, Th17, and T_{reg}-driving cytokines and supplemented with increasing concentrations of DETA-NONOate. The graph presents the mean ± SD from one representative experiment (triplicate cultures) of two (different healthy donors). (E and F) Relative gene expression of IL-17A (log scale) and Rorc (log scale), and secretion of IL-17A by naive (E) or naive and memory (F) $CD4^+$ T cells, expanded with anti-CD3/CD28 antibodies in the absence or presence of Th17-driving cytokines and NO donor (DETA-NONOate). The graphs present the mean ± SD from one representative experiment (E: triplicate cultures; F: quadruplicate cultures). The results were confirmed in 3 independent experiments using cells of different healthy donors. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

5.4.2 Cytokine- or MDSC-driven Th17 differentiation depends on the induction of endogenous NOS2 in naïve $CD4^+$ T cells and elevation of endogenous NOS2 in memory $CD4^+$ T cells

Unexpectedly, we observed that Th17-driving cytokines, as well as exogenous NO, all induce the expression of endogenous NOS2 in the expanding population of naïve $CD4^+$ T cells (>99% pure at the day of analysis) and further enhance its expression in memory Th cells (Fig. 5.4, A-C). A similar induction of endogenous NOS2 was also observed in blood-isolated naïve and memory

$CD4^+$ T cells, as well as tumor-isolated TILs, differentiated in the presence of cancer-isolated MDSCs (*data not shown*).

To eliminate the possibility that such enhanced NOS2 levels in expanding T cell cultures resulted from contamination with rare non-T cells expressing very high levels of NOS2, we evaluated the presence of intracellular NOS2 protein in individual $CD4^+$ T cells. As shown in Fig. 5.4D, the addition of Th17-driving cytokines induced distinct elevation of NOS2 in individual differentiating T cells, with further enhancement of intracellular NOS2 observed in T cells differentiated in the additional presence of NO donor. Consistent with the enzymatic activity of the endogenous $CD4^+$ T cell-expressed NOS2, we observed intracellular accumulation of NO in the individual Th17-differentiated cells (Fig. 5.4E), which was completely blocked using two different small molecule inhibitors of NOS2 activity (pan-NOS-inhibitor and NOS2-selective inhibitor; Fig. 5.4E). In line with the requirement for endogenous NOS2/NO-signaling in the effective induction of human Th17 cells, blockade of the endogenous NOS2 in $CD4^+$ T cells differentiating in the presence of Th17-driving cytokines suppressed their ability to secrete IL-17A (Fig. 5.4F).

The induction of NOS2 in $CD4^+$ T cells was closely correlated with the activity of the individual Th17-driving factors (and their combinations) in inducing Th17 differentiation (Fig. 5.4G). IL-1β, recently shown to be the critical component of the Th17-promoting cytokine cocktail [424, 428], was sufficient to induce significant expression of NOS2 in $CD4^+$ T cells, but its combination with the additional Th17-driving cytokines (IL-6 and IL-23) was needed for the optimal induction of NOS2 (Fig. 5.4G). This effect was further amplified by exogenous NO (Fig. 5.4, B, D and E). In contrast to IL-1β, IL-6, and IL-23, which together promoted the elevation of endogenous NOS2 in human $CD4^+$ T cells (and associated IL-17A production), human TGF-β_1

proved to be a suppressor of both IL-17A and NOS2 (Fig. 5.4G). Unlike the potent induction of endogenous NOS2 in $CD4^+$ T cells driven toward Th17 development, neither NOS1 (undetectable) nor NOS3 expression were induced during this process (*data not shown*).

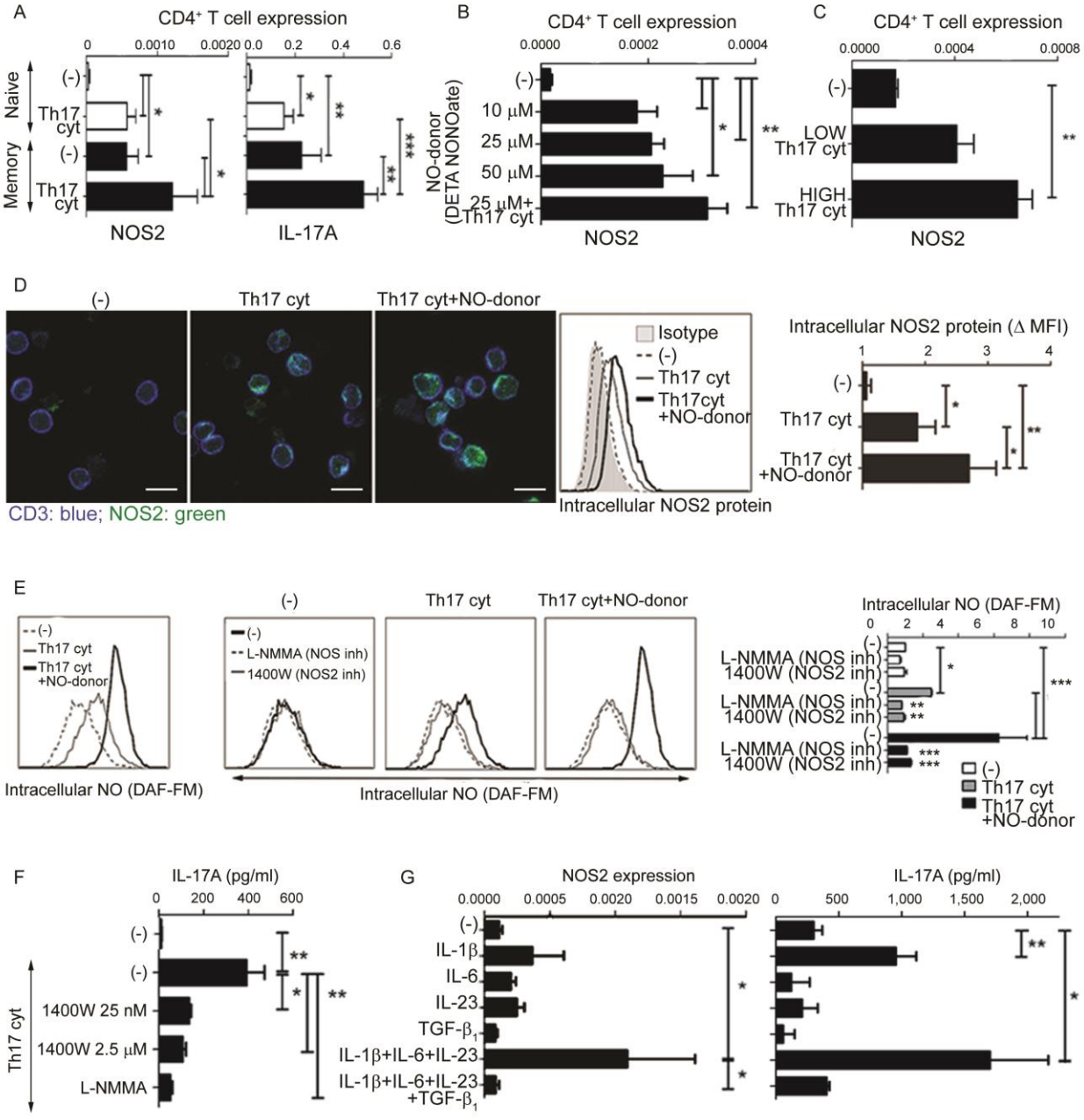

Figure 5.4. Endogenous T cell-expressed NOS2 and T cell-produced NO are required for *de novo* Th17 differentiation from naïve precursors and induction of the Th17 phenotype in memory cells.

(A) Comparative induction of NOS2 (left) and IL-17A (right) gene expression in naïve and memory $CD4^+$ T cells (mean ± SD from 3 healthy donors) stimulated with anti-CD3/CD28 antibodies in the absence or presence of Th17-driving cytokines. (B) Dose-dependent induction of NOS2 gene expression in naive $CD4^+$ T cells stimulated with anti-CD3/CD28 antibodies in the presence of increasing concentrations of NO donor (DETA-NONOate) and Th17-driving cytokines. The graph presents the mean ± SD from one representative experiment (performed with triplicate cultures). The results were confirmed in 3 independent experiments using different healthy donors. (C) Dose-dependent induction of NOS2 gene expression in bulk $CD4^+$ T cells, stimulated with anti-CD3/CD28 antibodies and Th17-driving cytokines (high: 20 ng/ml IL-1β, 50 ng/ml IL-6, 10 ng/ml IL-23; low: 25x dilution). The graph presents the mean ± SD from one representative experiment (triplicate cultures). The results were confirmed in 3 independent experiments using different healthy donors. (D) Induction of intracellular NOS2 protein (left and middle: representative data, right: mean ± SD from n=3 healthy donors) in $CD3^+$-gated bulk $CD4^+$ T cells stimulated with anti-CD3/CD28 antibodies in the presence of Th17-driving cytokines and NO donor (DETA-NONOate). Scale bar represents 10 μM. Data in the right panel is represented as the fold change of the mean fluorescence intensity (MFI) over the isotype control. (E) Induction of intracellular NO (DAF-FM staining; representative experiment, left; mean ± SD from n=3 healthy donors, right) in $CD3^+$-gated bulk $CD4^+$ T cells stimulated with anti-CD3/CD28 antibodies in the presence of Th17-driving cytokines and NO donor (DETA-NONOate), or the presence of general NOS inhibitor (L-NMMA) or NOS2-specific inhibitor (1400W). Data in the right panel is expressed as the fold increase of DAF-FM MFI over $CD4^+$ T cells cultured in the absence of Th17-driving cytokines and NO donor. When not otherwise indicated, statistically significant differences compared to the absence of NO inhibitors are shown. (F) IL-17A secretion by naïve $CD4^+$ T cells stimulated with Th17-driving cytokines in the presence of general NOS inhibitor (L-NMMA) or NOS2-specific inhibitor (1400W). The graph presents the mean ± SD from one representative experiment (quadruplicate cultures). The results were confirmed in 3 independent experiments using different healthy donors. (G) Induction of NOS2 (left, mean ± SD from 4 healthy donors) gene expression correlated with the IL-17A production (right, mean ± SD from 3 healthy donors) in bulk $CD4^+$ T cells by the individual Th17-inducing factors IL-1β, IL-6, IL-23, and/or TGF-β_1. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Interestingly, the induced levels of endogenous NOS2 were much higher in human memory than in naïve $CD4^+$ T cells (Fig. 5.4A), consistent with the observed differences in the

effectiveness of IL-17 induction in these two populations (see ref. [151] and current Fig. 5.2, C and I, Fig. 5.3F, and Fig. 5.4A). However, very high differences in baseline NOS2 levels between human memory and naïve $CD4^+$ T cells (mean ± SD: naïve: 0.00003±0.00002 vs. memory: 0.0006±0.0002, p=0.0053) could not be seen in their murine counterparts, where both memory and naïve $CD4^+$ T cells expressed similarly high baseline levels of NOS2 (naïve: 0.0034±0.001 vs. memory: 0.0032±0.001, not significant), and were not significantly modulated in the presence of Th17-promoting cytokines (mouse naïve: 0.0048±0.001 vs. memory: 0.005±0.001, not significant). These observations are consistent with the previously-reported differences in the regulation of mouse and human immune system by NO [405-407], different susceptibility of human and mouse naïve $CD4^+$ T cells to Th17-inducing factors [151, 413, 414], and the recently-reported lack of positive impact of exogenous NO on mouse $CD4^+$ T cells at any concentrations of NO donor (ref. [400] *and current mouse data not shown*).

5.4.3 Persistent expression of endogenous NOS2 and persistent cGMP-signaling are required for the functional stability of Th17 cells: Reversal of established Th17 cells from cancer patients by NOS- and cGMP-inhibitors

Interestingly, NOS2 blockade in $CD4^+$ T cell cultures activated in the presence of Th17-driving cytokines revealed that endogenous NO is not only required for the induction of IL-17A and IL-17F expression, but also for the optimal expression of IL-23 receptor (Fig. 5.5, A and B), known to be essential for the maintenance of Th17 function [429]. Indeed, NO induced IL-23R expression on naïve $CD4^+$ T cells, which express significantly less IL-23R than memory $CD4^+$ T cells (Fig. 5.5B, left). In contrast to IL-23R, no impact of NO on IL-2R expression was observed (Fig. 5.5B, right). Furthermore, NOS inhibition reduced IL-23R expression by memory $CD4^+$ T

cells (Fig. 5.5B), suggesting the requirement for NO in the optimal delivery of IL-23-mediated signals, which may contribute to the persistence of the Th17 phenotype.

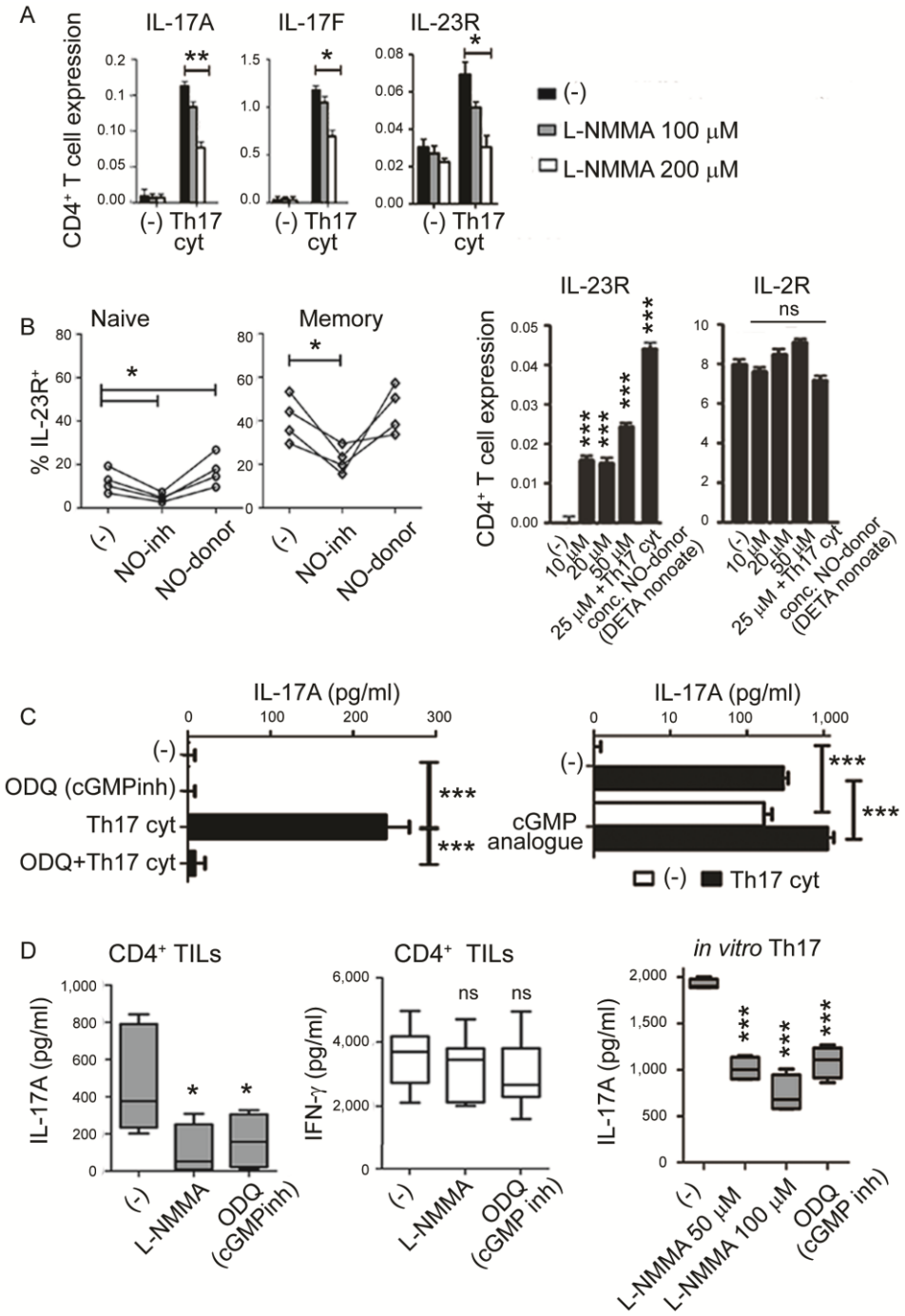

Figure 5.5. Endogenous NOS2 and persistent cGMP signaling are required for the NO-assisted *de novo* induction of Th17 cells and for the stability of human *in vivo*-developed Th17 cells from cancer patients.

(A) Relative gene expression of IL-17A, IL-17F, and IL-23R in bulk $CD4^+$ T cells, expanded with anti-CD3/CD28 antibodies in the absence or presence of Th17-driving cytokines and general NOS inhibitor (L-NMMA). The graphs present the mean ± SD from a representative experiment (triplicate cultures) of two (using different patients/healthy donors), which both yielded similar results. (B) Regulation of surface IL-23R expression (left) on naive and memory $CD4^+$ T cells (mean ± SD from 4 healthy donors) activated with anti-CD3/CD28 antibodies in the presence of NOS inhibitor (L-NMMA) or NO donor (DETA-NONOate); Relative gene expression of IL-23R and IL-2R (right) in naive $CD4^+$ T cells in the presence of increasing concentrations of NO donor and Th17-driving cytokines. Statistically significant differences compared to the absence of DETA-NONOate and Th17-driving cytokines are indicated. The graphs present the mean ± SD from a representative experiment (performed with triplicate cultures). The results were confirmed in 3 independent experiments using different healthy donors. (C) IL-17A production by naive $CD4^+$ T cells stimulated with anti-CD3/CD28 antibodies in the absence or presence of cGMP inhibitor (ODQ, left) or supplemented with cGMP analogue (Br-cGMP, right) in the absence or presence of Th17-driving cytokines. The graphs present the mean ± SD from a representative experiment (triplicate cultures). The results were confirmed in 3 independent experiments using different healthy donors. (D) IL-17A (left) or IFN-γ (middle) production by OvCa-isolated $CD4^+$ TILs (mean ± SD from 5 patients) expanded with anti-CD3/CD28 antibodies and re-stimulated in the absence or presence of NOS inhibitor (L-NMMA) or cGMP inhibitor (ODQ) for 48 h (statistically significant differences compared to the absence of inhibitors are indicated). IL-17A production by *in vitro*-generated Th17 cells (right; generated in 8 d cultures of $CD4^+$ T cells stimulated with anti-CD3/CD28 antibodies in the presence of Th17-driving cytokines), pretreated or not for 48 h with NOS inhibitor (L-NMMA) or cGMP inhibitor (ODQ). The data are shown as mean ± SD from 4 healthy donors. Statistically significant differences compared to condition in the absence of inhibitors are indicated. ns: $P>0.05$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

NO has been shown to signal predominantly via the cGMP/cGK signaling cascade [430], while high concentrations of NO involve the additional NO-dependent modification of a wider spectrum of endogenous proteins as well as nonspecific cytotoxic effects [401, 431]. In order to define the pathway of NO signaling relevant to Th17 induction, and to identify new targets for therapeutic modulation of Th17 responses and Th17-dependent pathologies, we evaluated the

role of cGMP/cGK signaling in these phenomena. Our data show that the cGMP-specific inhibitor ODQ blocks IL-17A production in naive $CD4^+$ T cells activated in the presence of Th17-driving cytokines, while the addition of a membrane-permeable cGMP analogue alone can induce IL-17A production and further increase the production of IL-17A induced by Th17-driving cytokines (Fig. 5.5C).

Both NO and the cGMP signaling cascade proved to be required for the stability of established Th17 cells that developed in cancer patients (OvCa TILs) *in vivo* or were generated from healthy donors *in vitro*, since inhibition of either NOS or cGMP selectively suppressed IL-17A but not IFN-γ production by these cells (Fig. 5.5D). These data indicate that targeting of NO-activated cGMP/cGK signaling can be evaluated for therapeutic intervention in Th17-mediated disorders.

5.5 DISCUSSION

Prompted by the correlation between the local expression of IL-17A and NOS2 observed in the tumor environment of patients with ovarian cancer, we tested the role of the NOS2/NO/cGMP/cGK pathway in the development of Th17 cells from human naïve, memory, and tumor-infiltrating $CD4^+$ T cells. We observed that endogenous NOS2 activity and intracellular NO production induced in $CD4^+$ T cells by previously-identified Th17 inducers (IL-1β, IL-6, and IL-23) or by cancer-infiltrating MDSCs is critically required for the *de novo* induction of Th17 cells *in vitro* and for the stability of *in vivo*-arising Th17 cells from cancer patients. While, in accordance with previous reports [389, 390, 400, 402], high concentrations of exogenous NO non-selectively inhibited immune activation, including the proliferation and

differentiation of Th17 cell precursors, the levels of NO produced by myeloid cells and, endogenously, by $CD4^+$ T cells supported the induction of Th17 responses and were essential for the functional stability of Th17 cells. These results help to explain the heterogeneous and often paradoxical effects of NO and Th17 cells in the regulation of inflammation, autoimmunity, and cancer [389].

Interestingly, while MDSCs suppress naive $CD8^+$ T cell proliferation and acquisition of cytolytic functions (Fig. 5.1 and [26, 228, 234, 382]), they do not impair naive $CD4^+$ T cell proliferation, but instead promote the *de novo* induction of Th17 cells, an effect that may explain the paradoxic generation and presence of inflammatory Th17 cells in the immunosuppressive cancer-associated environment [392, 393, 416, 418]. Our data also indicate that in analogy to the positive feedback loop between Th1 cells and IFNγ-producing NK and $CD8^+$ T cells [280], Th17 cells not only promote NO-dependent effector mechanisms of immunity [411], but also benefit from interaction with NO-producing cells (neutrophils, macrophages, MDSCs), leading to the establishment of a positive feedback between such NO- and IL-17-producing 'effector' and 'helper' cells.

The impact of Th17 cells on cancer progression has been shown to be highly context-dependent, varying across different cancers and in different mouse models [432-436]. Their preferential ability to be attracted to tumor sites and subsequently promote local recruitment of other inflammatory cells or convert to Th1 cells can lead to the enhancement of local antitumor immunity in particularly advanced tumors [416, 437-440], which may explain the positive correlation between intratumoral Th17 numbers and local production of IL-17 with survival in OvCa patients [416, 434]. However, in several models, Th17 cells have also been shown to drive early tumorigenesis by promoting chronic inflammation, DNA damage, and tumor-associated

angiogenesis, as well as by exhibiting potent suppressive activity [441-446]. Further studies are needed to understand the regulation and functional relevance of these different Th17 activities in the tumor environment.

Our observations demonstrating that the stability of Th17 function requires endogenous NOS2 and that the induction of endogenous NOS2 in $CD4^+$ T cells benefits from the synergy between the previously-identified Th17-driving cytokines (IL-1β, IL-6, and IL-23; Fig. 5.4G) help to explain the paradox that while the synergy between IL-1β and IL-6 (or other Stat3 inducers) is sufficient for the effective induction of Th17 cells [424, 447], IL-23 signaling is an important component of the development of the Th17 phenotype and Th17 functions in most models [151, 412-415, 419-421]. Similar to previous reports [414, 415], we observed that TGF-β_1 suppresses the development of human Th17 cells (Fig. 5.4G), an effect that is mirrored by the ability of TGF-β_1 to suppress endogenous NOS2 in $CD4^+$ T cells. These data suggest that the differences in the relative importance of TGF-β_1 in the development of mouse Th17 cells in different models [412, 420-423] may need to be evaluated in the context of its impact on the production of endogenous (T cell-derived) and exogenous (produced by MDSCs and other myeloid or stromal cells) NO, which may potentially differ in different settings. Likewise, fundamental differences between mice and humans regarding NOS activity, NO production, and NO regulation ([405-407] and current data) may explain different requirements for TGF-β_1 in the development of Th17 cells in mice versus humans. Interestingly, mouse Th17 cells induced by TGF-β_1 and IL-6 have been shown to suppress T cell effector functions, while mouse Th17 cells differentiated with IL-1β, IL-6, and IL-23 are not immunosuppressive [448], highlighting the difficulties in cross-interpreting the results of mouse and human studies involving the interplay of Th17 cells and cancer.

The current data and the previously-reported importance of COX2/PGE_2 in the induction and stability of NOS2 production by MDSCs [234], as well as the ability of NO to enhance COX2 activation [449], indicate a close interplay between these two inflammatory systems, which may provide new insights into the mechanism of the COX2/PGE_2-driven development of Th17 responses to different pathogens [450-452] and help to identify potential new therapeutic targets. Similarly, it remains to be seen if such TLR ligands as LPS or peptidoglycan, shown to be particularly effective in promoting the DC-mediated induction of human Th17 cells [151, 414], are particularly effective in inducing DC production of NO or alternative activators of the cGMP/cGK-mediated signaling pathway. Whether potential differences in this regard may contribute to the different efficiency of induction of Th17 cells from naïve versus memory and effector precursors ([151] and the current data) deserves further exploration.

Our data suggest that the previously-observed differences in the ability of human memory and naïve $CD4^+$ T cells to develop into Th17 cells (see ref. [151] and current Fig. 5.2C and Fig. 5.3F) may, at least partially, result from much higher baseline levels of endogenous NOS2 in human memory $CD4^+$ T cells, compared to naïve $CD4^+$ T cells (Fig. 5.4A). Our preliminary data indicate that a similar difference does not exist in the mouse system, where both naïve and memory cells express very high baseline levels of NOS2, and do not further elevate its levels during Th17 differentiation (*data not shown*). These differences in the baseline levels of NOS2 between human and mouse cells and the requirement for inflammatory factors in the expression of NOS2 by human cells are consistent with the significant delay in the demonstration of the presence of NOS2 in human cells [453-455] and its cloning [455].

The current identification of NO, NOS/NOS2, and the cGMP/cGK-signaling pathway as critical requirements for the induction and stability of human Th17 cells, both arising *in vivo* in

cancer-bearing patients and induced *in vitro* from naïve and memory precursor cells from healthy donors, suggests a number of potential therapeutic strategies. These strategies include inhibition of NO production or signaling in Th17-dependent malignant tumors or in Th17-mediated inflammatory/autoimmune processes, or the activation of these pathways to boost desirable Th17 immunity in Th17-susceptible tumors or chronic infections, such as *M. tuberculosis.* Since Th17 cells have a high propensity to migrate to and accumulate in tumor lesions, the current demonstration that NOS2 blockade can revert Th17 cells into cells preferentially producing IFN-γ suggests that the sequential application of NO donors or NO-increasing factors *in vivo* (or *ex vivo* to prepare tumor-homing T cells for adoptive immunotherapy) followed by systemic NOS2 inhibition (to promote their transition from Th17 to Th1 cells) may result in particularly high therapeutic effectiveness, promoting T cell accumulation at tumor sites and their subsequent conversion to type-1 effector cells. The feasibility of such approaches is enhanced by the availability of large numbers of NO donors and inhibitors that have been evaluated for the treatment of autoimmune and inflammatory diseases, as well as for vasodilation in hypertensive coronary disease, erectile dysfunction, and pulmonary hypertension.

6.0 ACTIVATED NK CELLS INDUCE TUMOR-ASSOCIATED SUPPRESSIVE FEEDBACK THROUGH THE COX2/PGE_2-DRIVEN ENHANCEMENT OF MDSCS

Adapted from:

Enhancement of the COX2/PGE_2 axis drives the self-limiting nature of type-1 immunity in cancer tissues

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These data are reported as a Manuscript in Preparation.

6.1 ABSTRACT

Type-1 immune responses are essential for effective anti-tumor immunity. Despite recent advances in the induction and stabilization of these responses by cancer immunotherapies, the clinical success of these approaches remain limited. Here, we report that the activation of type-1 immunity within the human tumor environment initiates IFNγ- and TNFα-dependent counter-regulation, driven by amplification of prostaglandin E_2 (PGE_2) and the key regulator of PGE_2 synthesis, cyclooxygenase 2 (COX2). We demonstrate that activated NK cells and CTLs induce IFNγ/TNFα-mediated over-expression of indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS/NOS2), IL-10, and COX2 by tumor-associated myeloid-derived suppressor cells (MDSCs). Importantly, this self-limiting suppressive feedback driven by type-1 immunity could be eliminated not only by neutralization of IFNγ and TNFα, factors critically required for the anti-tumor activity of immune effector cells, but also by COX2 blockade, which counteracted the IFNγ/TNFα-driven enhancement of all other suppressive factors. Our data demonstrate an intrinsic mechanism driving the self-limiting nature of type-1 immunity within the human tumor environment, and provide rationale for targeting the COX2/PGE_2 axis as part of cancer immunotherapies.

6.2 INTRODUCTION

Type-1 immunity, characterized by the development of cytotoxic $CD8^+$ T cell (CTL), natural killer (NK) cell, and type-1 helper $CD4^+$ T cell (Th1) responses producing the key cytokines IFNγ and TNFα, have been shown to be essential for effective anti-tumor immunity [21]. Driven

by the strong positive prognostic relevance of intratumoral type-1 lymphocytes to clinical outcome, current cancer immunotherapies focus on enhancing the accumulation, activation, and function of these lymphocytes within the tumor environment [456]. Nevertheless, despite progress in enhancing intratumoral type-1 immune processes, the clinical success of these approaches often remain limited to a small proportion of patients [457, 458].

The highly immune-suppressive nature of the tumor environment has emerged as a critical regulator of intratumoral immune responses, and is increasingly recognized as a major barrier to the effectiveness of cancer immunotherapies [459]. This includes the recruitment and induction of significant suppressive myeloid populations within the tumor environment, including a profound enrichment of myeloid-derived suppressor cells (MDSCs) [231]. Characterized in human cancer by a $LIN^{-}CD11b^{+}CD33^{+}HLA\text{-}DR^{low/-}$ phenotype, MDSCs have been demonstrated to potently inhibit both innate and adaptive immune responses through such key mechanisms as inducible nitric oxide synthase (iNOS/NOS2), indoleamine 2,3-dioxygenase (IDO), and IL-10 [244, 265, 408, 460].

Here, we demonstrate that the induction of potent suppressive counter-regulation is a direct consequence of type-1 immune activation within the human tumor environment. We identify the hyper-activation of MDSCs by type-1 lymphocytes as a key mediator of this feedback immune suppression, driven critically by amplification of prostaglandin E_2 (PGE_2) and its key synthesizing enzyme cyclooxygenase 2 (COX2). Blockade of the COX2/PGE_2 axis was capable of reversing the suppressive enhancement induced by type-1 lymphocytes through the central antagonism of multiple suppressive processes. These data indicate a key pathway for the induction of negative feedback immune suppression induced by type-1 responses within the

human tumor environment, and provide rationale for the core targeting of COX2/PGE_2 in uncoupling immunity and suppression for cancer therapy.

6.3 MATERIALS AND METHODS

Media and reagents. Bulk ovarian cancer ascites cells, MDSCs, NK cells, and T cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum and 1% L-glutamine and penicillin/streptomycin (all from Gibco, Invitrogen). The following factors were used in this study: IL-18 (200 ng/ml; MBL International); IFNα (1000 IU/ml; Intron A, IFN-α-2b; Schering-Plough); IL-12 (5 ng/ml; PeproTech); IL-2 (250 IU/ml; Chiron); granulocyte macrophage colony-stimulating factor (1000 IU/ml; GM-CSF); IFNγ (1000 IU/ml; Miltenyi Biotech); and TNFα (50 ng/ml; Miltenyi Biotech). The following inhibitors were used in this study: celecoxib (20 μM; BioVision), 1-methyl-DL-tryptophan (1 mM; Sigma-Aldrich), L-NMMA (200 μM; Cayman Chemical), anti-IL-10 mAb (clone 25209; 1 μg/ml; R&D Systems), and nor-NOHA (200 μM; Cayman Chemical). The concentrations used did not affect viability in cell cultures, as confirmed by live cell counts.

Isolation of ovarian cancer (OvCa) ascites cells. Human OvCa ascites were obtained intraoperatively from previously-untreated patients with advanced (stage III or IV) epithelial ovarian cancer undergoing primary surgical debulking for clinical staging. Written informed consent was obtained prior to any specimen collection, and the nature and possible consequences of the studies were explained. All specimens were provided under a protocol approved by the University of Pittsburgh Institutional Review Board (IRB0406147). Primary OvCa ascites cells were harvested by centrifugation. When indicated, bulk OvCa ascites cells were stimulated with

combinations of IL-18, IFNα, IL-12, IL-2, anti-CD3 mAb (clone OKT3; 1 μg/ml; eBioscience), and CD3/CD28 Human T cell-Activator Dynabeads (5 μl/ml; Invitrogen). NK cells were depleted from bulk OvCa ascites cells by CD56 positive magnetic selection (StemCell Technologies). MDSCs were depleted or isolated using CD11b positive magnetic selection (Miltenyi Biotech), which was previously shown to be highly effective in isolating >95% pure $CD11b^+$ cells uniformly expressing the $LIN^-CD11b^+CD33^+HLA\text{-}DR^{low/-}$ MDSC phenotype from human OvCa ascites cells [27, 234]. Control $CD11b^+$ cells were isolated from health donor peripheral blood using the same method.

Isolation of NK cells and $CD8^+$ T cells. Peripheral blood from healthy donors was harvested by venipuncture under IRB-approved protocols. NK cells ($CD56^+CD3^-$) and naïve $CD8^+$ T cells ($CD8^+CD45RA^+CCR7^{high}CD45RO^-CD56^-CD57^-$) were isolated by negative magnetic selection (>95% pure in both cases) using the EasySep system (StemCell Technologies), according to the manufacturer's protocol.

MDSC activation. For NK cell activation of MDSCs, NK cells ($0.5x10^5$ cells/well) were co-cultured with MDSCs ($1x10^5$ cells/well) in 96-well plates in the presence of IL-18 (200 ng/ml) and IFNα (1000 IU/ml). When indicated, soluble decoy receptors to IFNγ (sIFNγR1; 10 μg/ml; R&D Systems) and TNFα (sTNFR1; 1 μg/ml; R&D Systems) were added to cultures at co-culture initiation. For $CD8^+$ T cell activation of MDSCs, T cells ($0.5x10^5$ cells/well) were co-cultured with MDSC ($1x10^5$ cells/well) in 96-well plates in the presence of anti-CD3 mAb (1 μg/ml) and IL-12 (5 ng/ml). Alternatively, MDSCs were cultured with IFNγ (1000 IU/ml) and TNFα (50 ng/ml) to induce activation. Cultures were assessed at 24 h for mRNA expression and at 36 h for intracellular staining and ELISA analysis of supernatants.

$CD8^+$ T cell suppression. Naïve $CD8^+$ T cells ($1x10^5$ cells/well) labeled with CFSE (Invitrogen; labeled according to the manufacturer's protocol) were stimulated with CD3/CD28 Human T cell-Activator Dynabeads (5 µl/ml; Invitrogen) in the presence or absence of MDSCs ($0.25x10^5$ cells/well) and/or IL-18/IFNα-activated NK cells ($0.25x10^5$ cells/well) in 96-well plates, in the additional presence of small-molecule inhibitors or blocking antibodies against suppressive factors where indicated. On day 4, expanded $CD8^+$ T cells were analyzed for proliferation via CFSE dilution and intracellular granzyme B expression.

Flow cytometry. Cell surface and intracellular immunostaining analyses were performed using an Accuri C6 Flow Cytometer. NK cells and T cells were stained with the dye-conjugated anti-human mouse monoclonal antibodies CD56-PE-Cy5 (Beckman Coulter), CD3-PE (eBioscience), CCR7-FITC (R&D Systems), granzyme B-PE (Invitrogen), and CD16-FITC, CD8-PE-Cy5, CD45RA-FITC, CD45RO-PE, and CD57-FITC (BD Biosciences). MDSCs were stained for CD11b-FITC, CD14-PE, CD33-APC, CD34-PE-Cy5, and HLA-DR-PE (BD Biosciences and eBioscience), as well as IDO-A488 (R&D Systems), NOS2-PE (Santa Cruz Biotechnology), and COX1-FITC/COX2-PE (BD Biosciences). The corresponding mouse antibody isotype controls IgG1-FITC, IgG2b-FITC, IgG1-PE, IgG2a-PE, IgG1-PE-Cy5, IgG1-APC, and IgG1-A488 (BD Biosciences) were used, as appropriate. Before staining, the cells were treated for 20 min at 4ºC in PBS buffer containing 2% human serum, 0.5% BSA, 0.1% NaN_3, and 1 µg/ml of mouse IgG (Sigma-Aldrich) to block non-specific binding. Cell permeabilization for intracellular staining was performed using the Foxp3 Fix/Perm Buffer Set (eBioscience), according to the manufacturer's protocol. Cells were stained for 40 min at 4ºC followed by washing with PBS buffer containing 0.5% BSA and 0.1% NaN_3, then fixed and stored in 4% paraformaldehyde until analysis.

ELISA. Supernatants from 36 h co-cultures of NK cells and MDSCs were analyzed for IL-10 by indirect sandwich ELISA using specific matched primary and biotinylated-secondary antibody pairs (R&D Systems), as previously described [234].

Quantitative real-time PCR. Analysis of mRNA expression was performed using the StepOne Plus System (Applied Biosystems), as previously described [173], using inventoried primer/probe sets. Expression of IFNγ, TNFα, IDO1, NOS2, IL-10, and/or COX2 was assessed 24 h following bulk OvCa ascites cell activation or following MDSC activation with type-1-activated lymphocytes or IFNγ/TNFα. The expression of each gene was normalized to HPRT1 and expressed as fold increase ($2^{-\Delta CT}$), where $\Delta C_T = C_{T\ (target\ gene)} - C_{T\ (HPRT1)}$.

Statistical analysis. Data was analyzed using unpaired and paired t tests (two-tailed) and one-way and two-way ANOVA, where appropriate. Significance was judged at an α of 0.05. Where indicated, data from multiple different patients and control donors are recorded as means (± SD) from n different donors, described in the figure legends. Data from representative experiments are presented as means (± SD) from triplicate cultures, and confirmed in multiple independent experiments, described in the figure legends.

6.4 RESULTS

6.4.1 Activation of type-1 immune cells in the human tumor environment promotes local immune suppression through the hyper-activation of MDSCs

While intratumoral type-1 immunity has been linked to favorable prognosis for the vast majority of cancers [181, 461], therapeutic strategies targeting the enhancement of these responses have

demonstrated only limited activity for most patients [457, 458], suggesting that type-1 responses within the tumor environment may promote counter-regulatory mechanisms restricting the overall development of anti-tumor activity. Using bulk cells isolated from the malignant ascites of patients with late-stage epithelial ovarian cancer, we stimulated these cells with factors known to induce type-1-polarized lymphocyte activation [150, 297, 326], including IL-18/IFNα and anti-CD3/IL-12 to activate NK cells and $CD8^+$ T cells, respectively, as a model for type-1 immune activation within the human tumor environment. While, as expected, these stimuli induced high expression of the signature type-1 cytokines IFNγ and TNFα implicated heavily in the promotion of anti-tumor responses [462, 463], the same type-1-driving factors also induced significant expression of the known suppressive factors IDO1, NOS2, IL-10, and COX2 (Fig. 6.1A). This was found to be a general consequence of type-1 activation within the bulk tumor environment, as a similar enhancement in the expression of these suppressive factors was observed upon ascites cell treatment with several other known type-1-driving NK cell and T cell stimuli (Appendix Fig. 6).

Using IL-18/IFNα-driven NK cell activation as a model, we found that this enhancement in suppressive factors induced by type-1-driving stimuli was indeed due to lymphocyte activation within the tumor environment, as prior NK cell depletion abrogated this effect (Fig. 6.1B), while no influence on suppressive factor expression was observed after NK cell depletion in the absence of activation stimuli (data not shown). We have previously identified the profound enrichment of a monocytic subset of MDSCs, known to be a potent producer of these suppressive factors, within the human ovarian cancer tumor environment [27, 234]. Hypothesizing that these cells may be involved in this phenomenon, we depleted MDSCs from the bulk ascites cell population (see Material and Methods) prior to treatment with NK cell-

activating stimuli, and observed a strong reduction in the expression of suppressive factors induced by NK cell activation (Fig. 6.1C). Collectively, these results suggest that type-1-activated lymphocytes may promote immune-suppressive molecules within the tumor environment through the augmentation of MDSC activity.

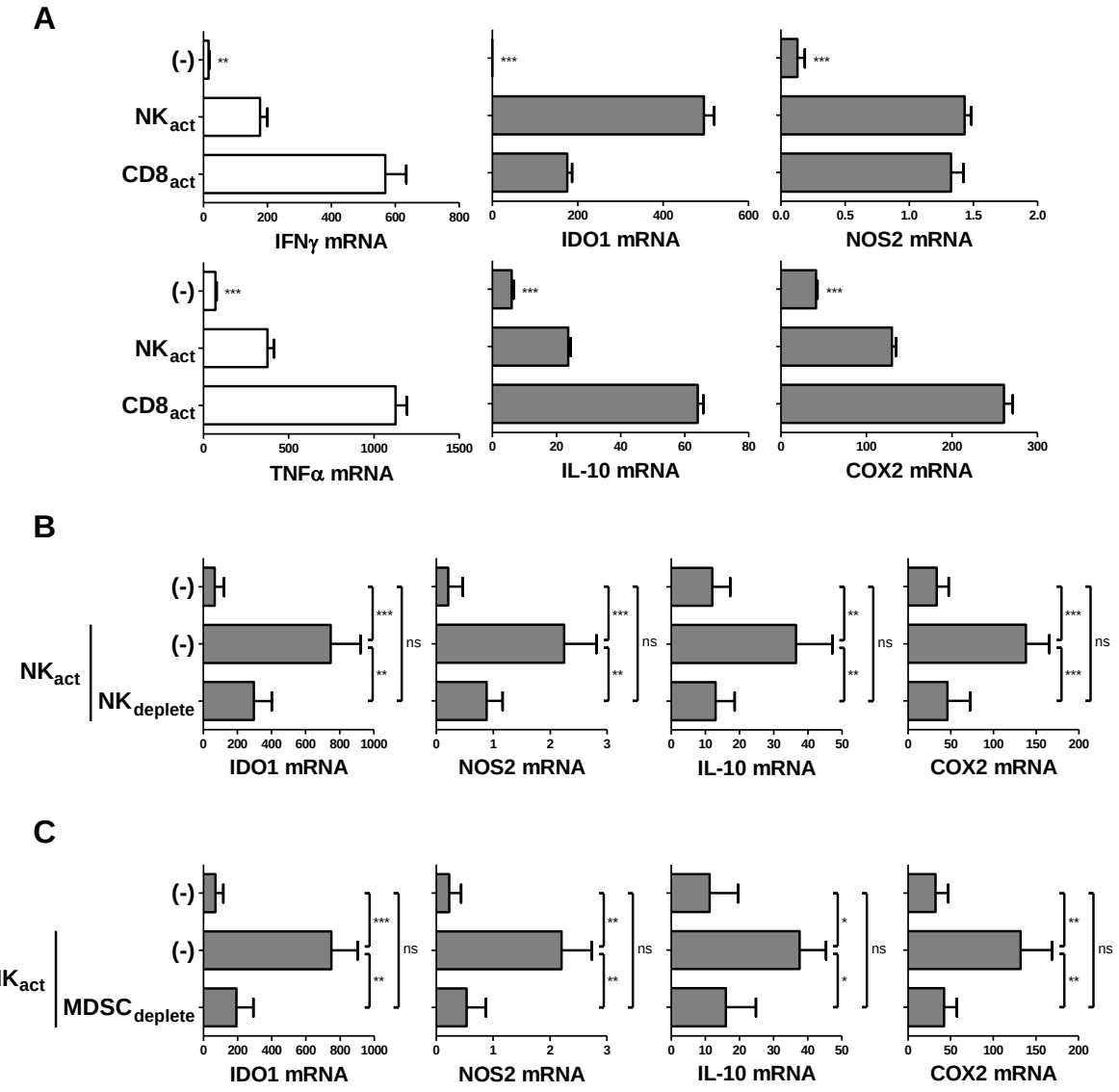


Figure 6.1. Type-1 activation of immune cells within the bulk tumor environment enhances local MDSC-mediated expression of suppressive factors.

(A) Bulk OvCa ascites cells were cultured for 24 h in the absence or presence of the NK cell-activating (NK_{act}) stimuli IL-18/IFNα or the $CD8^+$ T cell-activating ($CD8_{act}$) stimuli anti-CD3/IL-12, and analyzed for expression of IFNγ, TNFα, IDO1, NOS2, IL-10, and COX2. Data are expressed as ratios between the expression of individual genes and HPRT1, and shown as the mean expression (± SD) of triplicate cultures. Data represent one of three independent experiments, all yielding similar results. (B) Bulk OvCa ascites cells or ascites cells depleted of $CD56^+$ NK cells ($NK_{deplete}$) were cultured for 24 h in the absence or presence of the NK cell-activating (NK_{act}) stimuli IL-18/IFNα, and analyzed for expression of IDO1, NOS2, IL-10, and COX2. Data are expressed as ratios between the expression of individual genes and HPRT1, and represent the mean (± SD) of 4 independent patients. (C) Bulk OvCa ascites cells or ascites cells depleted of $CD11b^+$ MDSCs ($MDSC_{deplete}$) were cultured for 24 h in the absence or presence of the NK cell-activating (NK_{act}) stimuli IL-18/IFNα, and analyzed for expression of IDO1, NOS2, IL-10, and COX2. Data are expressed as ratios between the expression of individual genes and HPRT1, and represent the mean (± SD) of 3 independent patients. ***$p<0.001$, **$p<0.01$, *$p<0.05$, ns: $p>0.05$ compared to indicated groups or compared to all groups when not specifically indicated.

Indeed, direct co-culture of activated NK cells and isolated MDSCs significantly enhanced the ability of MDSCs to suppress the proliferation and granzyme B acquisition of naïve $CD8^+$ T cells driven by anti-CD3/CD28 antibodies (Fig. 6.2A) or autologous DCs (data not shown), an effect that was not observed with NK cell-activated control $CD11b^+$ myeloid cells isolated from the peripheral blood of healthy donors (Appendix Fig. 7). This heightened MDSC suppressive activity was accompanied by enhanced MDSC expression of IDO1, NOS2, IL-10, and COX2 at both the mRNA (Fig. 6.2B) and protein (Fig. 6.2C) levels, and a similar augmentation of MDSC suppressive factors was likewise observed following MDSC co-culture with type-1-activated $CD8^+$ T cells (Appendix Fig. 8). No suppression was observed by activated NK cells alone (Fig. 6.2A), which did not express IDO1 and COX2 and only low levels of NOS2 and IL-10 (data not shown). These data indicate that enhanced suppressive activity is mediated by MDSCs activated by direct interaction with type-1 lymphocytes.

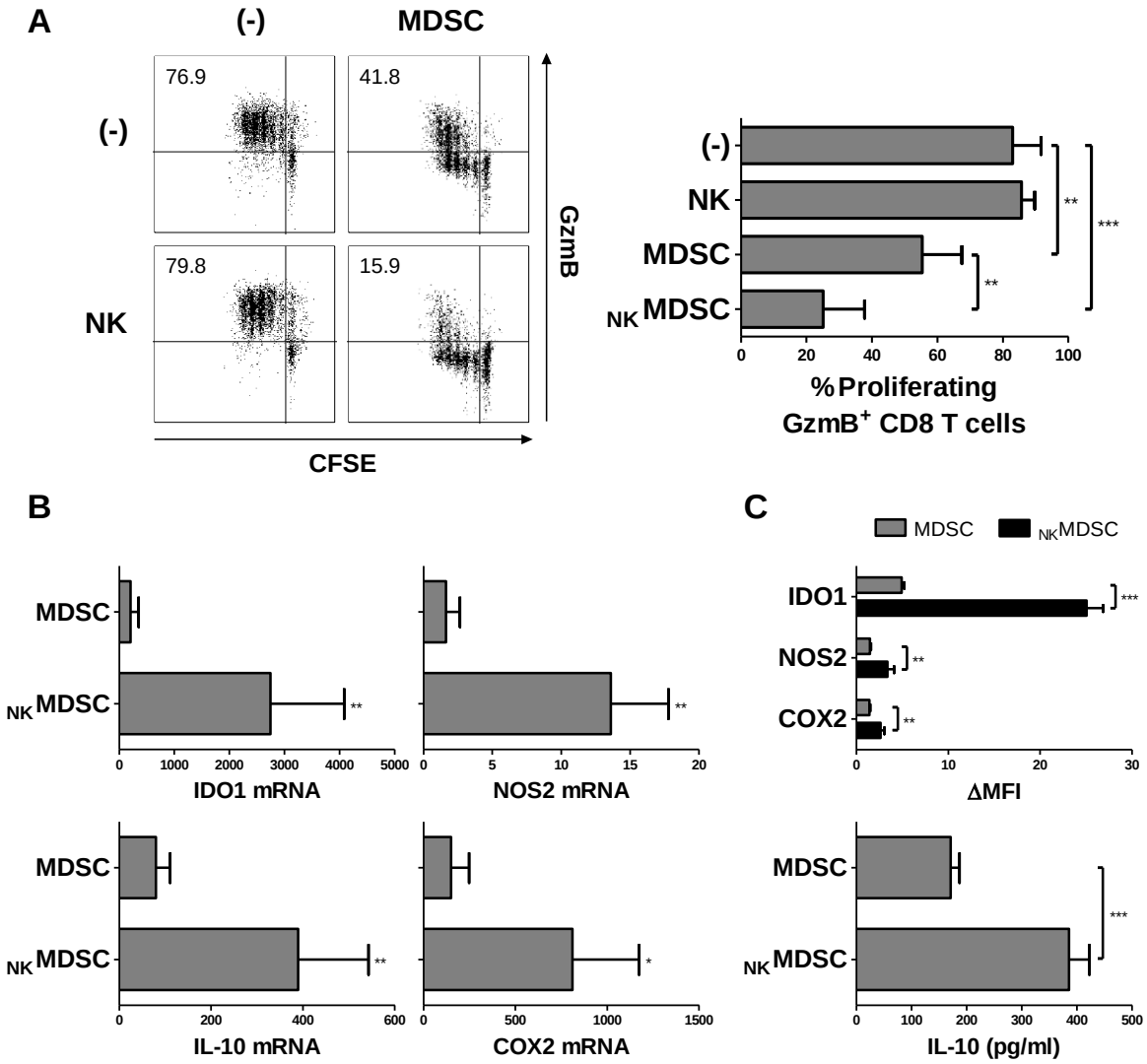


Figure 6.2. Activated type-1 immune effector cells enhance MDSC suppressive activity.

(A) Percentage of proliferating, granzyme B (GzmB) positive naïve $CD8^+$ T cells following 4 d activation with anti-CD3/CD28 antibodies in the absence or presence of OvCa ascites-isolated $CD11b^+$ MDSCs and/or IL-18/IFNα-activated NK cells ($_{NK}$MDSC), measured by CFSE dilution and intracellular GzmB staining presented in representative cultures (left) or as the mean (± SD) of 5 independent patients (right). (B-C) Expression of IDO1, NOS2, IL-10, and COX2 assessed by mRNA (B) and protein (C) levels in MDSCs cultured (24 h for mRNA; 36 h for protein) with or without IL-18/IFNα-activated NK cells ($_{NK}$MDSC). Data of mRNA levels are expressed as ratios between the expression of individual genes and HPRT1, and represent the mean (± SD) of 4 independent patients. Data of protein levels are represented as the fold change of the mean fluorescence intensity (ΔMFI) over the isotype

control, or levels detected by specific ELISA in 36 h supernatants, shown as the mean levels (± SD) of triplicate cultures. Data represent one of three independent experiments, all yielding similar results. ***p<0.001, **p<0.01, *p<0.05 compared to indicated groups or compared to all groups when not specifically indicated.

6.4.2 Key role of IFNγ and TNFα in the enhanced MDSC suppression induced by type-1 immune effector cells

Blockade of IFNγ and TNFα with soluble decoy receptors in MDSC co-cultures with NK cells significantly reduced the MDSC expression of multiple suppressive factors (Fig. 6.3), revealing the key role of these cytokines in mediating the enhanced MDSC suppressive activity. Indeed, treatment of MDSCs with exogenous IFNγ and TNFα mirrored the high expression of these suppressive factors seen after co-culture with type-1-activated lymphocytes (Appendix Fig. 9).

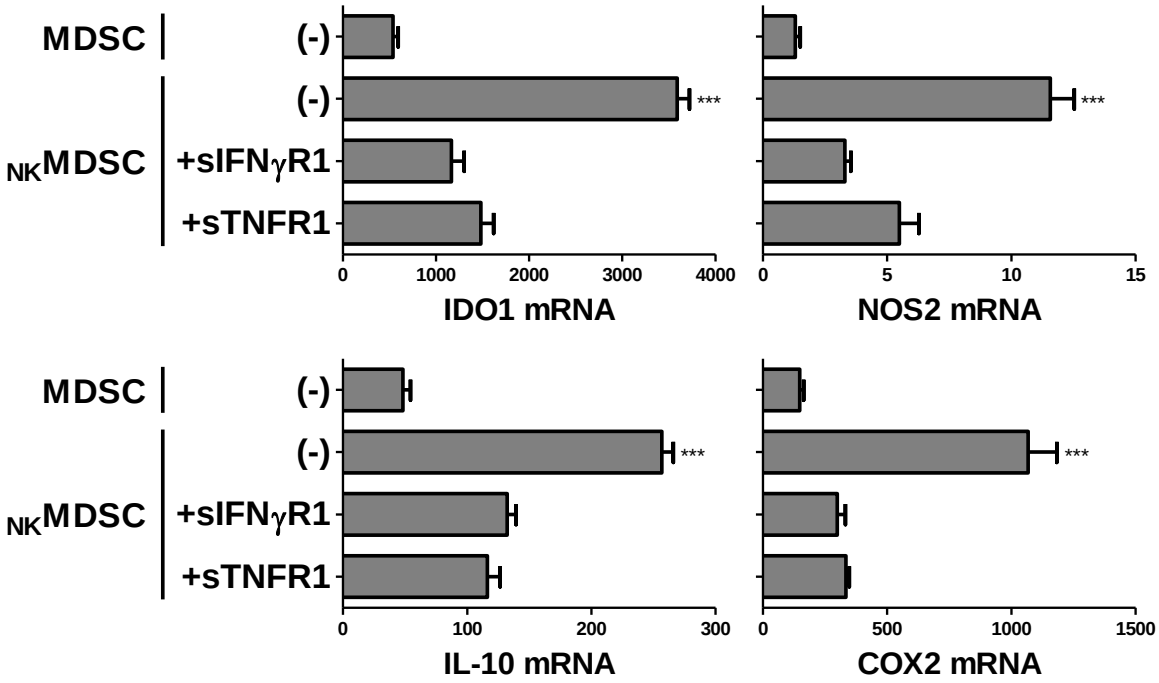


Figure 6.3. IFNγ and TNFα are critical mediators of MDSC hyper-activation induced by type-1 immune cells.

Expression of IDO1, NOS2, IL-10, and COX2 in MDSCs cultured for 24 h with or without IL-18/IFNα-activated NK cells ($_{NK}$MDSC), in the additional presence or absence of soluble IFNγ (sIFNγR1) or TNF (sTNFR1) decoy receptors. Data are expressed as ratios between the expression of individual genes and HPRT1, shown as the mean expression (± SD) of triplicate cultures in one of three similar experiments. ***$p<0.001$ compared to all groups.

6.4.3 Enhanced MDSC activity triggered by type-1 immune activation in the tumor environment requires the intact COX2/PGE_2 axis and is reversed by COX2 blockade

To investigate the key enhanced suppressive pathways used by MDSCs in the observed CTL suppression, we tested the effect of IDO, NOS, IL-10, arginase, and COX2 inhibition on the ability of 'hyper-activated' MDSCs to suppress $CD8^+$ T cell responses. Individual inhibition of IDO, NOS, IL-10, and arginase partially reversed the activation-induced MDSC suppression of $CD8^+$ T cell proliferation and granzyme B acquisition (Fig. 6.4A). Unexpectedly, however, sole inhibition of COX2 was significantly better than inhibition of any of the other pathways tested, even when blockade of all of these other pathways were combined (Fig. 6.4A). Analysis of NK cell-activated MDSCs revealed that COX2 blockade coordinately antagonized the expression of multiple other suppressive factors, as well as its own expression (Fig. 6.4B, left and middle). While PGE_2 has been described to have direct suppressive effects [464], these results suggest that COX2 inhibition's superior reversal of MDSC suppression is also likely to act through the regulation of other suppressive factors. Across multiple patients, COX2 blockade was capable of completely reversing the enhanced suppressive ability of hyper-activated MDSCs, which was restored upon exogenous PGE_2 supplementation (Fig. 6.4C). Notably, COX2 inhibition also increased the expression of IFNγ and TNFα in NK cell-MDSC co-culture (Fig. 6.4B, right), indicating the differential COX2-mediated modulation of stimulatory and suppressive factors.

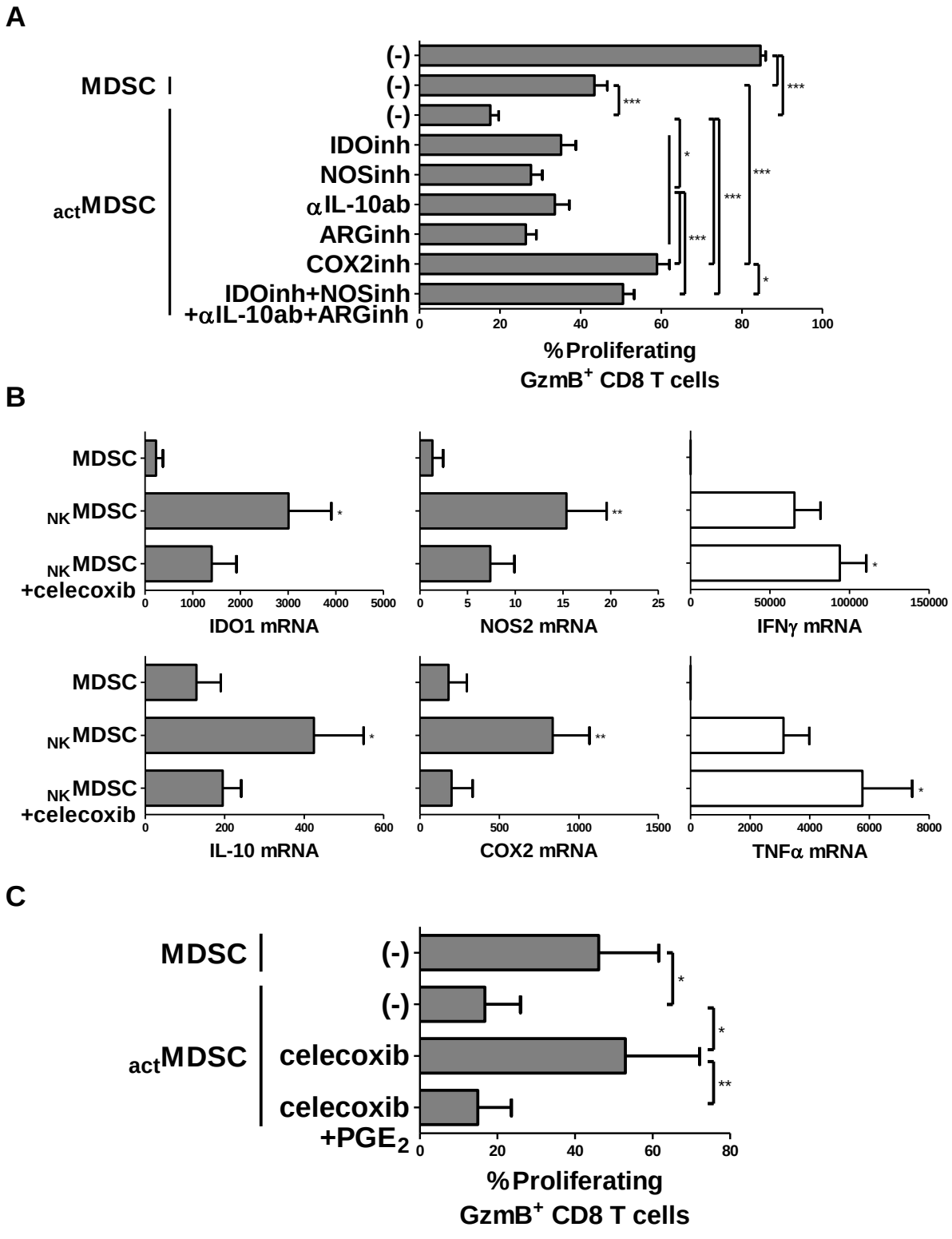


Figure 6.4. Effector cell-driven hyper-activation of MDSCs requires the intact COX2/PGE_2 axis.

(A) Percentage of proliferating, granzyme B (GzmB) positive naïve $CD8^+$ T cells following 4 d activation with anti-CD3/CD28 antibodies in the absence or presence of resting or IFNγ/TNFα-activated MDSCs ($_{act}$MDSC), in the additional presence of 1-MT (IDO inhibitor), L-NMMA (NOS inhibitor), neutralizing anti-IL-10 mAb, nor-NOHA (arginase inhibitor), and/or celecoxib (COX2 inhibitor). (B) Expression of IDO1, NOS2, IL-10, COX2, IFNγ, and TNFα in MDSCs cultured for 24 h with or without IL-18/IFNα-activated NK cells ($_{NK}$MDSC), in the additional presence or absence of celecoxib (COX2 inhibitor). Data are expressed as ratios between the expression of individual genes and HPRT1, and represent the mean (± SD) of 4 independent patients. (C) Percentage of proliferating $GzmB^+$ naïve $CD8^+$ T cells following 4 d activation with anti-CD3/CD28 antibodies in the presence of resting or IFNγ/TNFα-activated MDSCs ($_{act}$MDSC), in the additional presence of celecoxib (COX2 inhibitor) and/or exogenous PGE_2. ***$p<0.001$, **$p<0.01$, *$p<0.05$ compared to indicated groups or compared to all groups when not specifically indicated. When comparison to a group of conditions is shown, the least significant level is indicated.

6.5 DISCUSSION

The current data demonstrate that type-1 immune cells and the key cytokines mediating their anti-tumor activities, IFNγ and TNFα [462, 463], directly promote counter-regulatory suppressive events within the human tumor environment through the COX2/PGE_2-driven amplification of MDSC activity and its coordinated enhancement of multiple suppressive pathways. Our data help to reconcile reports demonstrating both anti-tumor and pro-tumor activities of type-1 cytokines [463, 465, 466], and identify the COX2/PGE_2 pathway as a key target for the therapeutic separation of opposing stimulatory and suppressive outcomes induced by type-1 immunity within the human tumor environment.

While many suppressive pathways, including the IDO, NOS, and IL-10 mechanisms described here, have been implicated in tumor-associated immune dysfunction [467], these data indicate that upregulation of these suppressive pathways can be a direct consequence of type-1

immune responses within the human tumor environment. The upregulation of many of these suppressive factors have long been associated with inflammatory mediators such as IFNγ in physiologic settings, including for homeostatic T cell contraction following infection [468], control of autoimmune responses [469-471], and immunologic tolerance during pregnancy [472], findings which suggest that counter-regulatory suppression induced by type-1 immunity may be a mechanism of normal endogenous immune control preventing overactive responses. However, in the setting of cancer, this mechanism may be co-opted in the tumor environment to support tumor progression. Indeed, recent clinical evidence in melanoma demonstrated that an enhanced intratumoral type-1 immune signature correlated with clinical response to ipilimumab, but was also associated with expression of IDO1 [473], potentially limiting the magnitude of these responses. We identify here that the type-1 immune-mediated hyper-activation of MDSCs, which are profoundly enriched within the human tumor environment [26], is likely to play a key role in this process.

While activation of MDSCs by pro-inflammatory factors has been previous described [218, 237, 239], we identify here the novel central regulation of the enhancement of multiple MDSC suppressive pathways by type-1 immune-mediated potentiation of the COX2/PGE_2 axis (Fig. 6.4B). As COX2/PGE_2 has also been shown to be involved in the *de novo* induction of MDSCs [234, 474, 475] as well as their recruitment to the tumor environment [27, 476], and has been further implicated in numerous other tumor cell-intrinsic and microenvironmental cancer-promoting activities (reviewed in [464, 477]), the type-1 immune-mediated enhancement of the COX2/PGE_2 axis described here may result in an even-larger expansion of tumor environment-associated suppression and the reinforcement of a suppressive feedback loop, severely limiting spontaneous or therapy-induced type-1 responses.

Our data demonstrate that IFNγ and TNFα produced by type-1 lymphocytes are the primary enhancers of the observed immune suppression (Fig. 6.3). Nevertheless, these molecules have also been extensively demonstrated to be critical to the effectiveness of anti-tumor immunity [462, 463], limiting the possibility of the therapeutic blockade of these factors as a part of cancer therapy. Identification of COX2/PGE_2 as a central regulator of multiple suppressive pathways downstream of IFNγ and TNFα secretion provides a key therapeutic target to maintain the anti-tumor features of these type-1 cytokines while preventing suppressive consequences. Indeed, COX2 blockade strongly reversed hyper-activated MDSC suppression of $CD8^+$ T cells in co-culture (Fig. 6.4C), and was more effective even than the combined treatment with IDO, NOS, arginase, and IL-10 inhibitors (Fig. 6.4A), while preserving and even enhancing lymphocyte secretion of IFNγ and TNFα (Fig. 6.4B).

Despite recent FDA approvals of several new forms of immunotherapy, including sipuleucel-T (Provenge) for prostate cancer [478-484], and ipilimumab [485] and pegylated IFNα for melanoma [486, 487], only a limited proportion of patients benefit from these immune therapies, and many of the responding patients eventually progress. Recent demonstrations that cancer can progress even in the presence of 10-40% tumor-specific T cells in the blood of vaccinated patients [488, 489] highlight the need to promote entry and local effector functions of these T cells within tumor tissues, which may be further enhanced by the antagonism of suppressive feedback mechanisms. Since approaches such as CTLA4 blockade or PD1/PDL1 blockade are believed to promote the duration of anti-cancer immunity [490], it remains to be tested whether their effectiveness and the duration of their activity can be enhanced by simultaneous blockade of PGE_2 synthesis or responsiveness to PGE_2 using available inhibitors of PGE_2 synthesis and signaling (reviewed in [464]).

In summary, the current findings provide novel insights into the self-limiting nature of type-1 immunity in human cancer, and provide rationale for targeting the COX2/PGE_2 axis as a key part of therapeutic approaches seeking to enhance the magnitude and duration of type-1 immune responses within the human tumor environment.

7.0 SUMMARY AND INTERPRETATIONS

It has become clear that NK cells not only participate as cytotoxic effector cells in the anti-tumor immune response, but also provide complex non-cytotoxic immunomodulatory functions essential to the development of protective immunity. The work described here presents a unifying model describing these key NK cell helper functions in the development of DC-mediated adaptive immunity, and in particular, implicate NK cells as central initiators and promoters of these responses at multiple significant levels. Our data also demonstrate the presence of negative feedback mechanisms on immune activation that may be involved in the physiologic resolution of these NK cell-driven responses, but which exist and are amplified within the human tumor microenvironment as a significant barrier to effective, sustained immune control. These findings provide key insights into the development of anti-tumor immunity and have considerable translational implications for the enhancement of cancer therapy.

7.1 EMERGING MODEL

The data presented herein suggest that NK cells mediate unique helper functions that are regulated distinctly from their killing capacity, and which can be enhanced by the synergy between IL-18 and NK cell recognition of other early indicators of cellular distress, such as stress-associated ‘induced-self’ NKG2D ligands (Fig. 3.2 and Appendix Fig. 5) or IFNα (Fig.

2.1) elaborated early in viral infection or tumor development [101-103]. Helper NK cells activated in the periphery by signals associated with tissue damage, infection, or transformation can function as key initiators of the immune response, recruiting immature DCs through the elaboration of crucial chemokines, including CCR5 ligands (Fig. 3.1 and 3.3), in order to facilitate optimal antigen acquisition. Subsequent NK cell interaction with DCs drives the process of DC maturation, including upregulation of co-stimulatory molecules as well as CCR7-mediated homing capacity to secondary lymphoid tissue (Fig. 2.5 and Appendix Fig. 1). NK cell interaction also imprints on DCs the capacity to secrete key factors, importantly including IL-12 (Fig. 2.4), essential for the polarization of T cell responses toward type-1 immunity [150], as well as the chemokine CCL19 (Fig. 4.1), a factor critical in DC-mediated priming of naïve T cells and induction of recall responses by memory T cells [491]. Thus, helper NK cell interaction with DCs promotes all three signals essential for driving DC-mediated anti-tumor type-1 T cell immunity: antigen-specific 'signal 1', co-stimulatory 'signal 2', and immune-polarizing 'signal 3.' Furthermore, NK cell collaboration with DCs also promotes a chemokine environment in peripheral tumor sites conducive to the infiltration of primed type-1 effector cells, characterized by high expression of CXCR3 and CCR5 ligands (Fig. 3.5). This occurs without a concurrent enhancement in CCL22-mediated T_{reg} cell entry (Fig. 3.4), and thus provides comprehensive support for both the afferent and efferent phases of anti-tumor immunity.

DCs have been classically defined as sentinels of the immune system that are specifically designed for danger recognition and the initiation of needed immune responses [492]. However, in a larger context, the cooperation between NK cells and DCs described herein likely exists to provide more complete surveillance of evolving threats. Importantly, within the tumor, classic acute inflammatory danger signals may be limited or absent [493], providing an even-more

critical role for the NK cell detection of ‘missing-self’ and/or ‘induced-self’ (such as diminished MHC class I molecules or stress-induced ligands recognized by NK cell receptors like NKG2D) in the initiation of immune responses [357]. Thus, the sequential NK cell to DC to T cell stimulation model presented here may be a key paradigm for how immune responses are initiated particularly in the cancer context. Indeed, this pathway has been implicated in tumor control *in vivo* in several instances, in which tumor rejection was shown to be reliant on NK cell recognition of tumor, subsequent DC activation, and the eventual development of protective adaptive T cell immunity [202, 204, 294]. Our current data further support the concept that NK cell helper ability may need only to recognize certain subsets of susceptible tumor cells in order to generate broader anti-tumor immune responses. For instance, NK cell interaction with susceptible K562 tumor cells (Fig. 2.3) was effective in engendering more comprehensive adaptive responses via DC activation (Fig. 2.6). Consistent with this concept, mouse *in vivo* studies have indicated that initial NK cell recognition of susceptible targets, such as those with MHC class I deficiency, could lead to the subsequent development of Th1 and CTL responses against the parental MHC class I-sufficient tumor [294]. This helper NK cell ability to translate recognition of only a subset of tumor cells into a much larger and more comprehensive adaptive immune response presents an attractive pathway to target in the context of cancer therapy.

Our data also indicates that a negative-feedback mechanism exists following type-1 immune activation, shown here to be a general consequence of activated NK cells as well as T cells secreting IFNγ and TNFα, of which MDSCs can play a key role (Chapter 6). It is likely that such a mechanism may be a physiologic balance to prevent immune damage from prolonged over-active responses, as suggested by the role of type-1 immunity in enhancing suppressive pathways during T cell contraction following infection [468] and in the protection from

autoimmunity [469-471]. Indeed, the involvement of the COX2/PGE_2 axis as a central regulator of this mechanism (Fig. 6.4) is telling, as it has been implicated in chronic disease states to support tissue preservation and repair, helping to contain damage stemming from prolonged immunity [464]. Our data strongly suggests however that this mechanism is co-opted by human tumors to limit anti-tumor immune responses. This occurs via a profound enrichment of COX2-expressing MDSCs capable of mediating potent $CD8^+$ T cell suppression (Fig. 5.1 and 6.2), as well as the polarization of $CD4^+$ T cell responses toward Th17- rather than Th1-type functional immunity (Fig. 5.2), which is likely to be less effective in controlling cancer [21].

Thus, this work provides an emerging model to understand the roles that NK cell engagement with DCs and MDSCs play in initiating and modulating type-1 anti-tumor immune responses. This work also reveals the impact of NK cell interactions on critical chemokine networks governing naïve T cell priming and effector T cell infiltration into peripheral tissues, as well as important pathways restricting these responses. The mechanistic insights provided by these studies further identify new opportunities for the therapeutic modulation of these responses for cancer therapy, the implications of which are discussed below.

7.2 IMPLICATIONS FOR IMMUNOTHERAPY

The initial characterization of NK cells as specialized killers has evolved into a broader appreciation of the many cytotoxic and non-cytotoxic roles these innate cells play in the development of protective immunity [200, 300, 494]. Although NK cell effector activities, such as cytolysis and IFNγ secretion, are still intuitively assayed and discussed as a single non-specific phenomenon, an increasing body of evidence supports the concept that these cytotoxic

and non-cytotoxic NK cell functional activities are in fact distinctly governed. For instance, it has been shown that the localization and trafficking of IFNγ and TNFα occur in compartments that do not overlap with perforin, are processed by different secretory pathways utilizing distinct endosomal proteins, and result in distinctive patterns of non-polarized versus polarized secretion [495]. Further evidence suggests that distinct NK cell surface receptors, such as KIR2DL4 versus those recognizing target cell-expressed CD40/CD80, may also differentially mediate cytokine secretion versus cytotoxicity [496, 497], and other studies have shown that even the same NK cell activating receptor, such as 2B4, is capable of driving lytic and non-lytic activities through separate downstream signaling pathways [498].

In line with these observations, the results shown here (Fig. 2.1, 3.1, and 4.1) clearly indicate that these cytotoxic and non-cytotoxic activities are distinct and differentially-regulated, and have the potential to be selectively promoted by therapy. This is most clearly demonstrated by the dichotomous functional consequences on NK-DC interaction induced simply by the application of IL-18 versus IL-2 for NK cell priming, in which IL-18 promotes potent NK cell activation of DCs (Fig. 2.4 and 2.5) and polarization toward desirable anti-tumor type-1 immunity (Fig. 2.6), while IL-2 promotes efficient NK cell killing of DCs (Fig. 2.1), thereby potentially hindering the evolution of downstream adaptive responses. The feasible therapeutic separation of these distinct NK cell activities using specific cytokines along with precise temporal and/or locoregional application may allow the practical augmentation of specific NK cell functions at selective sites for more effective, targeted treatment.

Our results also highlight the need to comprehensively assess the potential outcomes of any proposed therapeutic strategy in the human tumor context. For instance, our current data indicate that even IL-18-driven enhancement of a particular NK cell function, such as the

nominally-'helper' secretion of IFNγ and TNFα during immune-stimulatory interaction with DCs (Chapters 2-4), can have opposing suppressive outcomes when interacting with tumor-associated MDSCs (Chapter 6). Understanding the mechanisms underlying these anti- and pro-tumor outcomes will enhance our ability to uncouple the desirable from undesirable effects of our therapeutic strategies. For instance, the disruption of the COX2/PGE_2 axis provides one such opportunity, dissociating the stimulatory effects of IFNγ and TNFα produced by IL-18/IFNα-driven NK cells from their suppressive enhancement of MDSCs (Fig. 6.4), thus providing a rationale for the concurrent therapeutic use of IL-18-driven, helper NK cell-targeting combinatorial adjuvants with COX2 blockade. Recent observations describing distinct receptors governing NK cell killing of DCs versus tumor cells (NKG2D-independent versus NKG2D-dependent) [499] offers another example, suggesting the possibility for therapeutic blockade of undesirable DC lysis while maintaining desirable tumor cell killing. Thus, expanding our knowledge of such mechanisms will provide valuable new targets for therapeutically maximizing the net positive effect on anti-cancer immunity.

From our data (Fig. 2.1, 3.1, and 4.1), it is clear that the specific pattern of NK cell activation is critical for the outcome of NK cell responses, which may be potentially modified by the therapeutic application of key agents such as IL-18. However, our basic mechanistic results indicate that the effects of IL-18 are only revealed upon stimulation with secondary factors (Fig. 2.2, 2.3, 3.2, and 4.1). For instance, the type-1-driving functions of IL-18 shown in the present work depended on co-stimulation with other pro-inflammatory factors. These factors included type I interferons, IL-12, IL-2, or IL-15 (Fig. 2.2, 3.2, and 4.1), or susceptible target cells expressing low levels of MHC class I and high levels of activating NK cell receptor ligands (Fig. 2.2, 3.2, and Appendix Fig. 5), factors which may or may not be adequately present

endogenously in patients with advanced cancer. Because of this clear dependence on the availability, and likely the character, of these co-activating stimuli, the effects of applied therapies will need to be understood in the larger *in situ* tumor context. Indeed, although IL-18 has been extensively described to play an important role in effective anti-tumor immunity via its key effects on IFNγ production, *in vivo* promotion of lymphocyte proliferation and cytotoxicity, and enhanced NK cell recognition of tumor targets [336, 337, 500], IL-18 has also been shown in some models to promote tumor metastasis and growth via enhanced endothelial adhesion, induction of tumor growth factors, and promotion of a regulatory NK cell subset overexpressing PD-L1 [338, 339, 501].

IL-18 synergy with different factors within the tumor microenvironment could potentially account for these conflicting findings, either in the absence of positive co-stimulatory factors or the presence of negative co-stimulatory factors, and may suggest the need to additionally modify co-activating factors for optimal anti-tumor effectiveness. These data suggest that concurrent application of IL-18 with IFNα may drive immune-stimulatory processes directly within the human tumor microenvironment (Fig. 3.5) or within tumor-associated lymph nodes (Fig. 4.4), which may be potentiated by additional COX2 blockade (Fig. 6.4). IFNα currently enjoys the advantage of extensive clinical experience for cancer therapy [502], and thus represents an attractive candidate for immediate translation of these findings into clinical cancer care. Nevertheless, expanded investigation of additional combinatorial candidates in place of, or even in addition to, IFNα will likely yield new opportunities for optimizing this approach. Already, our data indicate that poly-I:C has powerful synergistic effects with IL-18 and IFNα in promoting NK-DC cross-talk, contributing to significantly-enhanced DC maturation, IL-12 production, and tumor-specific CTL induction in the context of late-stage melanoma patients

(Fig. 2.5, 2.6, and Appendix Fig. 2). These findings are supported by others in both human *in vitro* [90, 313] and mouse *in vivo* [314-316] settings, and poly-I:C is currently under active investigation as a component of robust adjuvant approaches [493].

Another attractive implication of this work is the suggested ability to therapeutically promote potent feed-forward interactions between NK cells and DCs by well-selected stimulatory combinations. For instance, our data support an IL-18-driven feed-forward accumulation of both DCs and NK cells promoted by reciprocal stimulatory chemokine interactions involving CXCR3 and CCR5 ligands (Chapter 3). Others have also shown a positive feedback between DC-secreted IL-18 and NK cell-secreted HMGB1 in mutual activating interactions [503]. Indeed, these results help us place in proper therapeutic context reports indicating that NK cells many not infiltrate in high numbers into human tumors [504]. With regard to the therapy proposed here, and the model of NK cells as key immune initiators, activation of only a small number of NK cells may be amplified through productive NK-DC interaction to a much larger immune-stimulatory response, helping to overcome these limitations.

The current work has focused on the influence of NK cells on $CD8^+$ T cell responses, either the promotion of CTL activity via DCs (Fig. 2.6 and 4.3) or suppression of CTL responses via MDSCs (Fig. 6.2 and 6.4), due to the clear significance of such effector T cells to clinical outcomes in the cancer setting [461]. However, our work has also demonstrated the existence of a robust population of $CD4^+$ Th17 cells *in vivo* in the human ovarian cancer environment (Fig. 5.2), spontaneously promoted by MDSCs, while others have also demonstrated a notable enrichment of $CD4^+$ T_{reg} cells in the tumor environment [21]. Although the prognostic significance of these populations remain to be definitively confirmed, their direct influence on

intratumoral immune processes, as well as their indirect impact on the relative balance between Th1 and other types of immunity, are likely to considerably influence therapeutic outcomes [433, 505]. Thus, an important unanswered question is the effect differentially-activated NK cells or NK cell-activating stimuli directly applied to the bulk tumor environment may have on these important $CD4^+$ T cell populations. Our data has demonstrated that induction and stability of Th17 cells within the human tumor environment critically depends on NO, provided exogenously by tumor-infiltrating MDSCs (Fig. 5.2) and produced endogenously within T cells themselves (Fig. 5.4). Our work has also shown that activated NK cells can drive robust MDSC expression of NOS2, the inducible isoform of the NO synthases (Fig. 6.1-6.3). Likewise, we have demonstrated that activated NK cells participate in the key induction of the COX/PGE_2 axis (Fig. 6.1-6.4), an inflammatory pathway closely interacting with the NO system [449] and implicated in the development of Th17 responses [450, 451]. Interestingly, however, the COX/PGE_2 axis has also been associated with promoting T_{reg} cells in the tumor environment [506, 507], and our data also indicate that activated NK cells can induce high tumor-associated expression of IDO (Fig. 6.1-6.3), which may also promote T_{reg} cells [508] and/or control the Th17 versus T_{reg} balance [509-511]. Thus, the effect, and underlying mechanisms, of differentially-activated NK cells or therapeutic NK cell-activating agents on the regulation of these intratumoral $CD4^+$ T cell populations remains an important open question with obvious implications for the selection of optimal therapeutic approaches.

It has also become clear that the inability of the immune response to control cancer is not likely to represent a single defect in the immune process [512]. Thus, new therapeutic approaches will require targeting of multiple aspects of the immune response for truly effective outcomes, including facilitating innate responses at the initiation of immunity, the priming of

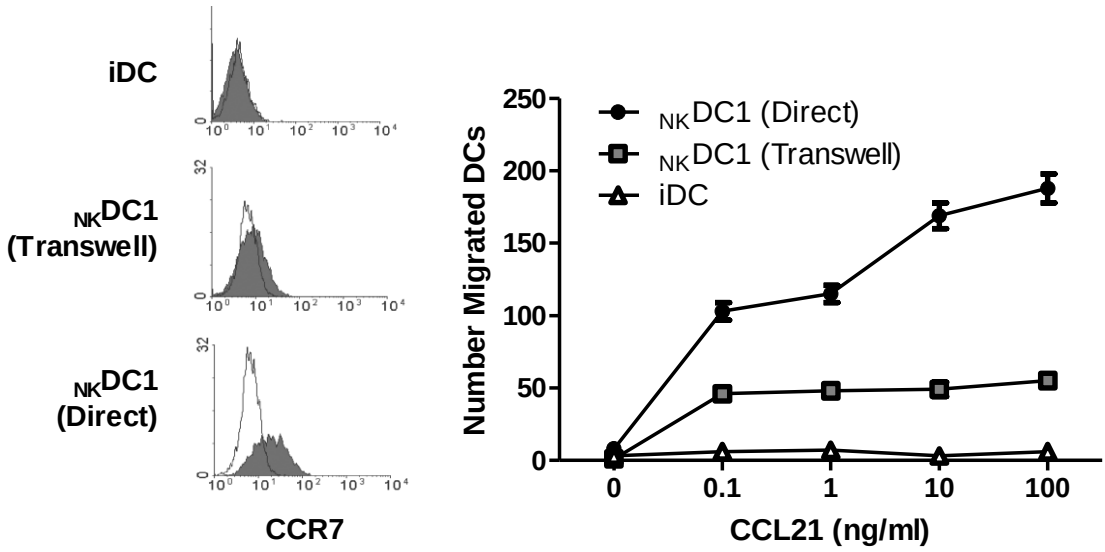
adaptive responses, and the infiltration of adaptive effector cells into the tumor microenvironment, as well as counteracting suppressive mechanisms. The therapeutic IL-18/IFNα-driven enhancement of NK cell helper function proposed here has the particular advantage of targeting multiple 'nodes' of the immune response simultaneously, including the accumulation of DCs at sites of tumor for antigen acquisition (Chapter 3); the activation, type-1 polarization, and LN-homing of DCs (Chapter 2); the enhancement of chemokine interactions necessary for T cell priming (Chapter 4); and the conditioning of the tumor environment for adaptive effector cell homing (Chapter 3). Nevertheless, this therapy will also likely benefit from combinatorial approaches, most especially for alleviation of the feedback immunosuppression associated with this immune activation (Chapter 6). Thus, the proposed approach may nicely synergize with therapies already in the clinic for cancer treatment, such as anti-CTLA4 and anti-PD1/L1 antibodies [490], or further therapies suggested by our work, including COX2/PGE_2 inhibitors and inhibitors of direct suppressive mediators (such as IDO, NOS2, and IL-10) (Fig. 6.4), which may further positively enhance the activity of NK cells, DCs, and T cells in this approach.

Finally, one of the greatest promises of immunotherapy is the ability to engender durable cancer regressions, such as the approximately 70% of complete responders to IL-2 therapy for melanoma and renal cancer with ongoing complete responses of more than 20-25 years [458]. The NK cell-targeted therapy suggested by our current findings may have similar prospects for durable responses. This includes the persistence of multiple NK cell-driven effects on CTL induction and cytokine and chemokine modulation extending beyond the initial NK-DC interaction (Fig. 2.4-2.6, 3.4, and 4.1-4.3), critically targeting the enhanced priming of DC-mediated adaptive T cell responses. Apart from classical adaptive memory, however, recent data

in the NK cell field has also suggested the intriguing possibility of an NK cell 'memory' phenotype. Existing both in mice [513, 514] and humans [515] *in vivo*, this NK cell 'memory' is characterized by long-term persistence of prior-activated NK cells and potent recall-type responses, retaining memory of the initial activation and responding robustly to re-challenge with secondary expansion and effector activity. Interestingly, induction of this phenotype in human NK cells *in vitro* [516] as well as for therapeutic cell-transfer in mice *in vivo* [517], including in the setting of established tumors [518], has suggested the critical involvement of IL-18 in association with IL-12 and IL-15, factors similarly implicated in the NK cell 'helper' effects described in our current work (Fig. 2.2, 3.2, and 4.1). Indeed, our data demonstrate that human NK cells can remember, at least within short-term cultures, their initial activation by IL-18, and can respond robustly to secondary stimuli even when the IL-18 stimulus has been removed (Fig. 2.2, 3.2, and 4.1), an effect we and others [196, 335] have described as NK cell 'priming'. It will be interesting to explore whether these 'priming' and 'memory' effects, apparently governed by many of the same signals, are simply kinetic descriptors or truly distinct but interrelated phenomena involving additional unique as-yet unidentified regulators. Such findings may provide novel approaches to further augment the durability of anti-tumor responses induced by NK cell activation, and ultimately enhance prospects for true cancer cure.

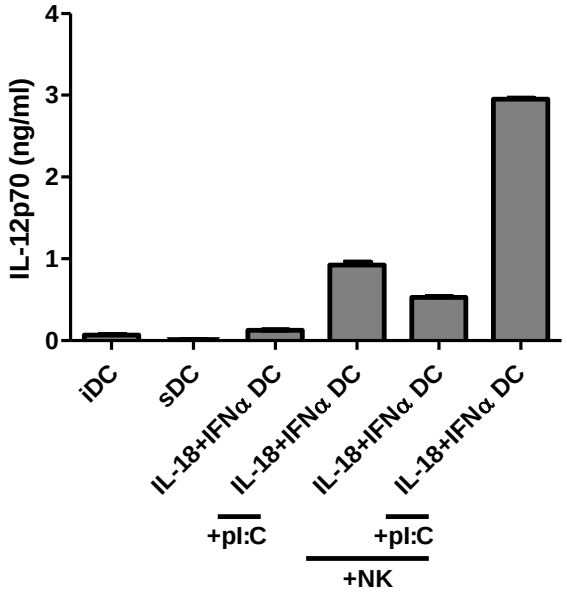
APPENDIX

SUPPLEMENTAL FIGURES



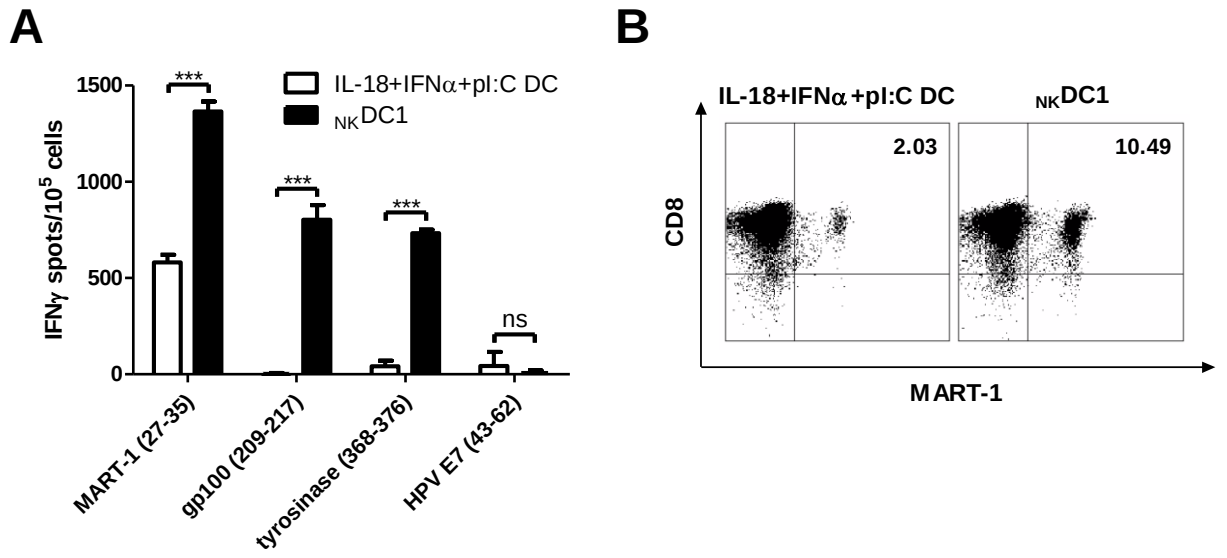
Appendix Figure 1. $_{NK}$DC1s have enhanced lymph node migratory capacity.

Surface expression of CCR7 (left; shaded histograms) and *in vitro* migration toward CCL21 (right) of untreated immature DCs (iDCs) or DCs treated with autologous NK cells and IL-18/IFNα/poly-I:C ($_{NK}$DC1s) in direct or transwell-separated co-cultures.



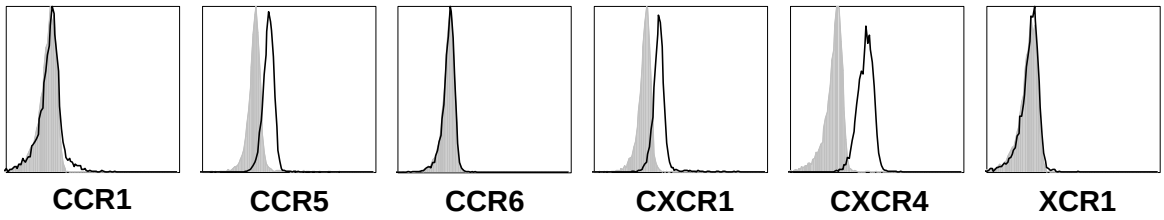
Appendix Figure 2. NK cells are required for the optimal induction of IL-12p70 production by DCs co-stimulated with poly-I:C.

IL-12p70 production by J558-CD40L-stimulated immature DCs (iDCs), DCs treated with the standard cytokine cocktail (sDCs), or DCs treated with IL-18/IFNα or IL-18/IFNα/poly-I:C with or without autologous NK cells. Data recorded as the mean (± SD) of triplicate cultures. Data shown was obtained from one representative experiment of three performed, all yielding similar results.



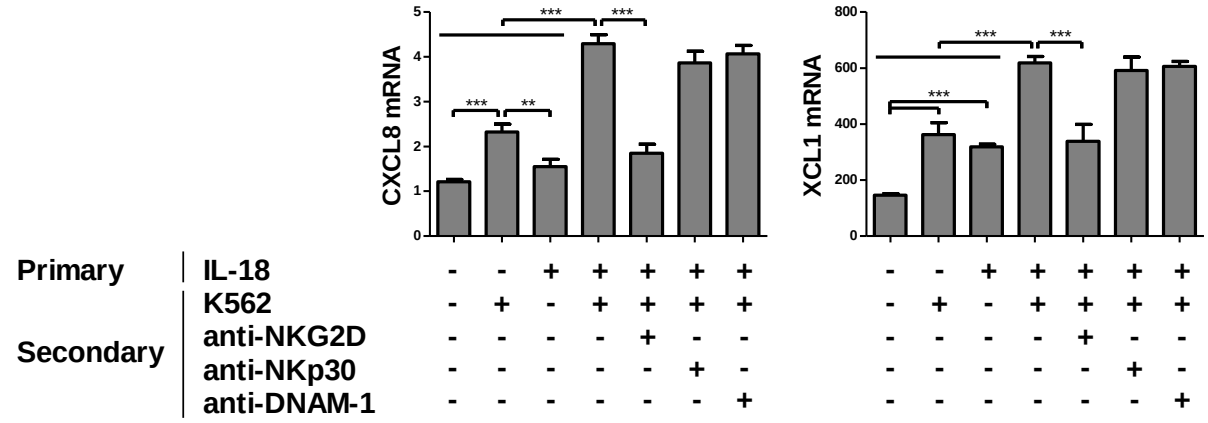
Appendix Figure 3. NK cells are required for the optimal induction of DC1s with a high capacity to induce melanoma-antigen-specific CTLs.

IL-18/IFNα/poly-I:C-stimulated DCs in the absence or presence ($_{NK}$DC1s) of autologous NK cells from HLA-A2$^+$ stage III and stage IV melanoma patients were pulsed with HLA-A2-restricted melanoma-associated peptides and used to sensitize autologous CD8$^+$ T cells. CTLs were assayed on day 24 of culture. (A) Frequencies of IFNγ-producing CD8$^+$ T cells responsive to T2 cells loaded with individual peptides, as determined by ELISPOT assay. Data recorded as the mean (± SD) of triplicate cultures. Data shown is from one representative experiment of three performed. (B) Flow cytometric analysis showing percentage of tetramer-positive MART-1-specific CD8$^+$ T cells generated through *in vitro* stimulation with melanoma peptide-pulsed, differentially-activated DCs. Inset numbers represent percent CD8$^+$MART-1$^+$ cells. Results from one representative experiment of three performed. ***$p<0.001$, ns: $p>0.05$ compared to indicated groups.



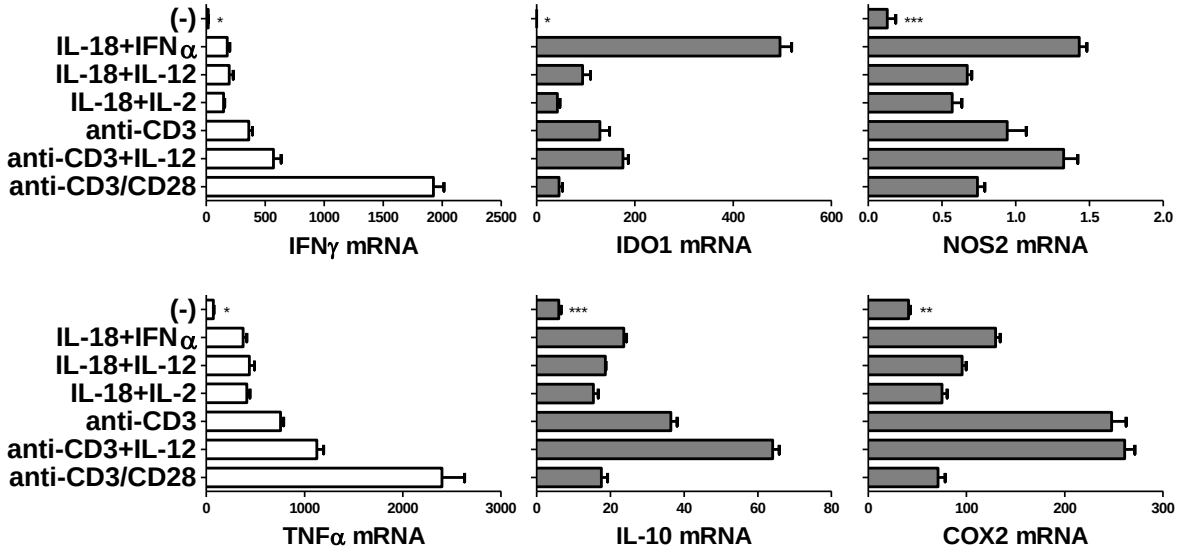
Appendix Figure 4. Chemokine receptor expression on peripheral blood-isolated DCs.

Representative surface expression (open histograms) of CCR1, CCR5, CCR6, CXCR1, CXCR4, and XCR1 on DCs isolated from healthy donor peripheral blood. Gray filled histograms represent isotype controls.



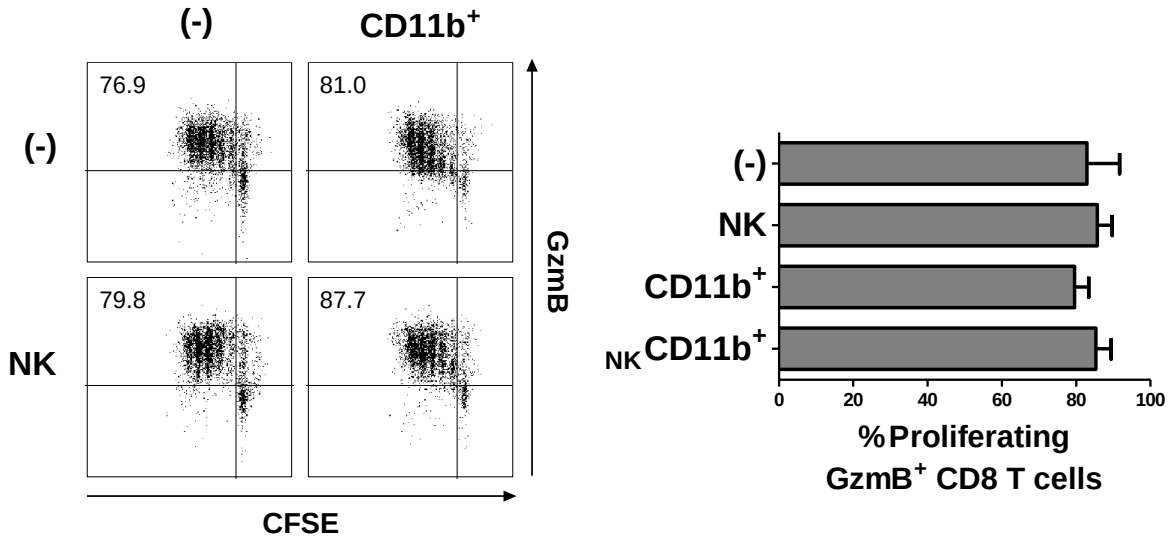
Appendix Figure 5. IL-18 synergizes with K562 tumor cell recognition in inducing NK cell expression of DC-attracting chemokines.

NK cells were pre-treated for 24 h in the absence or presence of IL-18, washed, and re-plated in the absence or presence of K562 cells (5:1 NK:K562 ratio). When indicated, NK cells were pre-treated for 30 min with blocking antibodies to NKG2D, NKp30, or DNAM-1 before co-culture with K562 cells. The expression of CXCL8 (left) and XCL1 (right) were analyzed after 4 h incubation with the secondary stimulus, and demonstrate a similar pattern to CCL3 and CCL4 (see Fig. 3.2B). Data are expressed as ratios between the expression of individual chemokine genes and HPRT1, and recorded as the mean expression (± SD) assayed in triplicate cultures. Data represent one of three independent experiments, which all yielded similar results. ***$p<0.001$ compared to indicated groups.

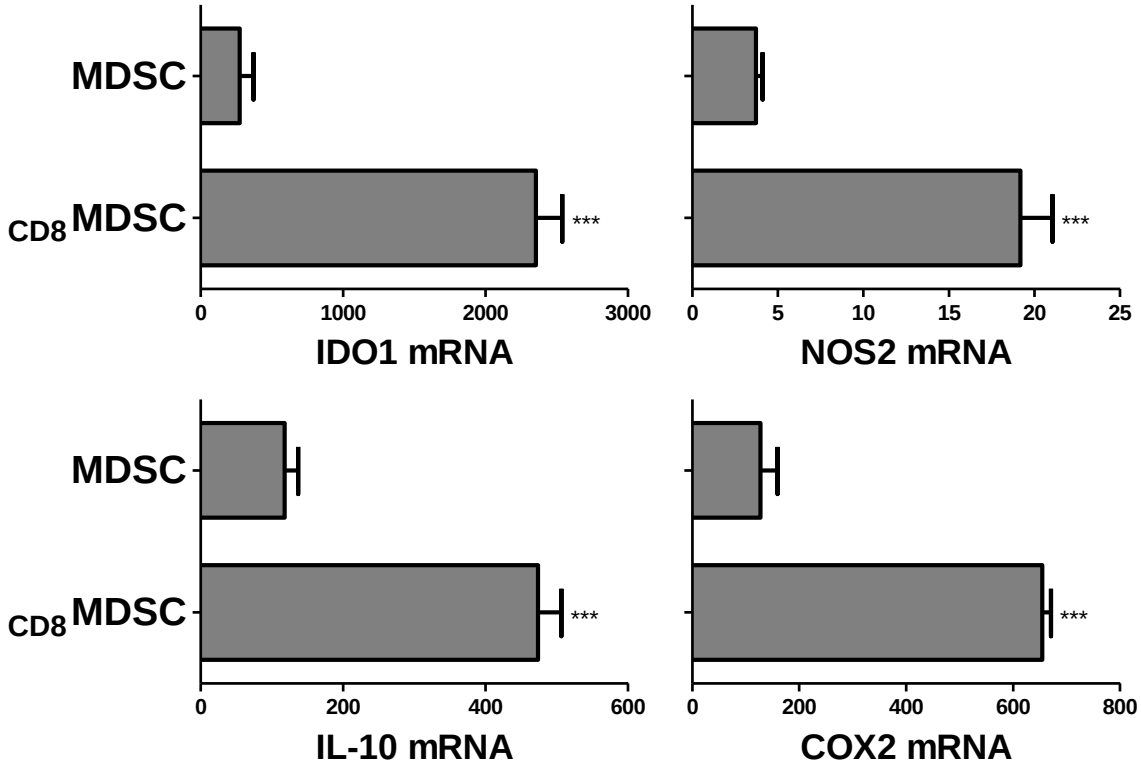


Appendix Figure 6. Lymphocyte activation by multiple stimuli within the bulk tumor environment enhances local expression of both stimulatory and suppressive factors.

(A) Bulk OvCa ascites cells were cultured for 24 h in the absence or presence of the indicated NK cell-activating or $CD8^+$ T cell-activating stimuli, and analyzed for expression of IFNγ, TNFα, IDO1, NOS2, IL-10, and COX2. Data are expressed as ratios between the expression of individual genes and HPRT1, and shown as the mean expression (± SD) of triplicate cultures. Data represent one of three independent experiments, all yielding similar results. ***$p<0.001$, **$p<0.01$, *$p<0.05$ compared to all groups.

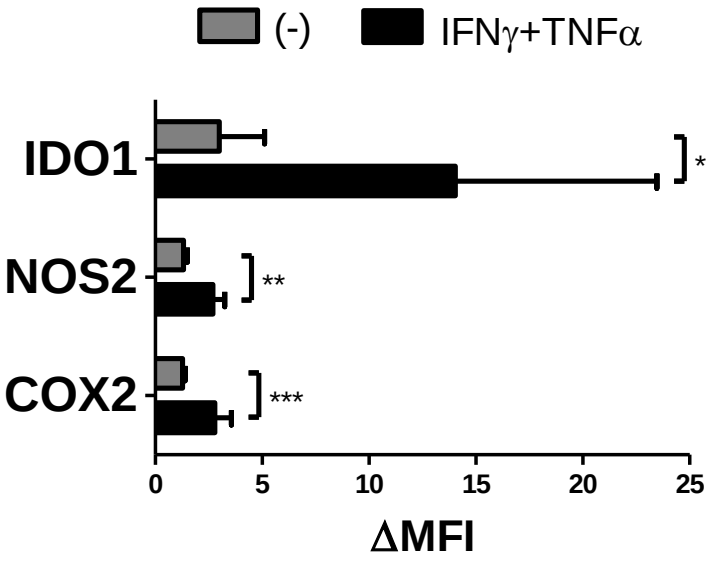


Appendix Figure 7. Type-1-activated NK cells do not enhance suppressive activity of control $CD11b^+$ cells. Percentage of proliferating, granzyme B (GzmB) positive naïve $CD8^+$ T cells following 4 d activation with anti-CD3/CD28 antibodies in the absence or presence of control $CD11b^+$ cells from health donor peripheral blood and/or IL-18/IFNα-activated NK cells ($_{NK}CD11b^+$), measured by CFSE dilution and intracellular GzmB staining presented in representative cultures (left) or as the mean (± SD) of 5 independent donors (right).



Appendix Figure 8. Type-1-activated $CD8^+$ T cells enhance MDSC expression of suppressive factors.

Expression of IDO1, NOS2, IL-10, and COX2 in MDSCs cultured for 24 h with or without anti-CD3/IL-12-activated $CD8^+$ T cells ($_{CD8}$MDSC). Data are expressed as ratios between the expression of individual genes and HPRT1, and shown as the mean expression (± SD) of triplicate cultures. Data represent one of two independent experiments, both yielding similar results. ***$p<0.001$ compared to other group.



Appendix Figure 9. IFNγ and TNFα drive enhanced MDSC expression of suppressive factors.

Expression of IDO1, NOS2, and COX2 protein in MDSCs cultured for 36 h with or without IFNγ/TNFα. Data are represented as the fold change of the mean fluorescence intensity (ΔMFI) over the isotype control, and represent the mean (± SD) across n independent patients (n=5 patients for IDO1, n=4 for NOS2, n=6 for COX2). ***$p<0.001$, **$p<0.01$, *$p<0.05$ compared to indicated groups.

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